BENNETT

AI-POWERED BIOLOGICAL INTELLIGENCE

IDENTIFYN RESOLUTION STEPDOWN

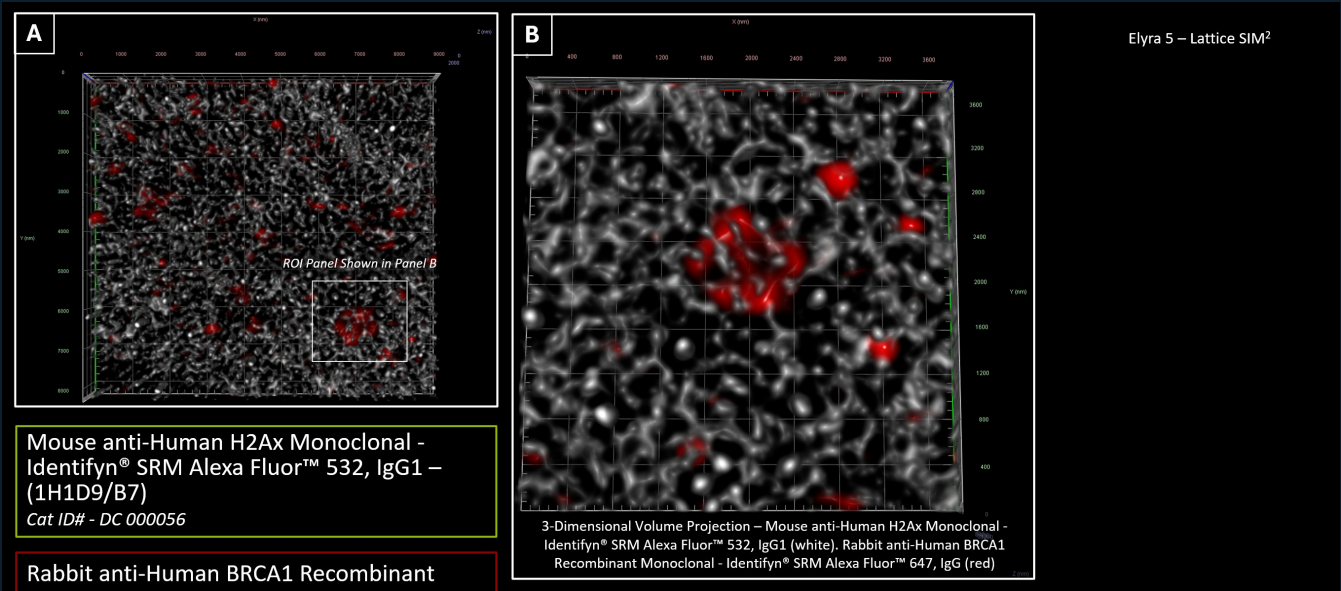
H2AX

MICROSCOPY EVIDENCE ASSESSMENT

Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532

WESTERN BLOT -> WIDEFIELD -> WIDEFIELD SIM / APOTOME -> CONFOCAL -> AIRYSCAN
SIM -> SIM²

7	0	PENDING
ORDERED EVIDENCE TIERS	SUPPORTING CONTROL	SCIENTIST REVIEW



Lattice SIM² imaging of the H2AX Alexa Fluor™ 532 Direct Conjugate, co-imaged with BRCA1 Alexa Fluor™ 647 following etoposide treatment, is shown as a 3D volume projection (Panel A) with the corresponding region of interest in Panel B. At ~40–60 nm lateral resolution, SIM² reveals increased chromatin-associated organization and BRCA1 spatial association relative to conventional SIM. Although discrete sub-chromatin subunits remain unresolved in the absence of single-molecule localization (STORM), SIM² provides the highest chromatin-level resolution achievable within this modality. Signal localization and organization are consistent with the unconjugated H2AX reference condition (Cat. ID# PA-MM-000002) and meet CDx go/no-go criteria for conjugation equivalency, localization specificity, and step-down advancement toward higher-resolution imaging.

REPRESENTATIVE HIGHEST-AVAILABLE VIEW / SIM²

DC-000056 | PRODUCT-LEVEL EVIDENCE DOSSIER

SOURCE-GROUNDED MICROSCOPY + BIOLOGICAL REVIEW / IDENTIFYN EVIDENCE PRESERVED

CRO CAPABILITY DEMONSTRATED

Identifyn performed the experimental and imaging work documented below. BENNETT converts that operating history into a governed, source-bounded scientific record.

- Source-documented sample preparation and antibody/reagent workflow
- Western blot imaging
- Widefield fluorescence microscopy
- Widefield structured illumination / Apotome optical sectioning
- Confocal microscopy
- Airyscan acquisition and reconstruction workflow
- Structured illumination microscopy
- SIM² reconstruction workflow
- Treatment-response experimental workflow
- Z-stack / 3D acquisition
- Multi-channel imaging

SCIENTIFIC QUESTION

What source-bounded experimental and microscopy evidence is preserved for H2AX, and what is the strongest interpretation allowed by the available modalities and controls?

EVIDENCE AVAILABLE

Western blot -> Widefield -> Widefield SIM — Apotome -> Confocal -> Airyscan -> SIM -> SIM²

BENNETT ASSESSMENT

The preserved DC-000056 record contains the applicable NIR sequence: Western blot; Widefield; Widefield SIM — Apotome; Confocal; Airyscan; SIM; SIM². The historical boundary reflects the validated application available for the specific conjugate, deployed platform and workflow, not a universal scientific impossibility.

BENNETT / MICROSCOPY AND BIOLOGICAL EVIDENCE REVIEW

SOURCE-GROUNDED RETROSPECTIVE REVIEW / PENDING SCIENTIST REVIEW

SOURCE STATE	SOURCE HASH VERIFIED / EVENT RECORDED
BENNETT EVIDENCE REVIEW	COMPLETED / SOURCE-BOUNDED
NATIVE IMAGE REPROCESSING	NOT PERFORMED
LITERATURE SOURCES	INDEPENDENTLY VERIFIED
SCIENTIST REVIEW	PENDING
PUBLICATION	NOT AUTHORIZED

OVERALL CALL / NIR-BOUNDED PARTIAL STEPDOWN

The preserved DC-000056 record contains the applicable NIR sequence: Western blot; Widefield; Widefield SIM — Apotome; Confocal; Airyscan; SIM; SIM². The historical boundary reflects the validated application available for the specific conjugate, deployed platform and workflow, not a universal scientific impossibility.

SETTINGS ASSESSMENT

The record preserves 7 ordered evidence tier(s). Structured source metadata values are reported by stage and remain tied to the original method objects.

BIOLOGICAL SUPPORT

The record supports retrospective, source-bounded microscopy review. Target identity, specificity, treatment effect, binding, interaction and mechanism are not inferred from presentation images alone.

CONTROL ASSESSMENT

No separate control record is preserved; none is inferred from method or template language.

CLAIM CEILING

This draft supports historical capability documentation, source registration, modality ordering, settings review and bounded interpretation. It does not independently validate antibody specificity, quantify a population response, establish binding, interaction, causation or mechanism, or authorize publication.

PROCESSING DISCLOSURE

Retrospective BENNETT architectural evaluation of preserved Identifyn source evidence. BENNETT did not perform native-file reprocessing, new deterministic measurement or the historical image post-processing represented in this record.

REAGENTS AND ACQUIRED CHANNELS

Preparation reagents are reported separately from channels actually documented in the acquisition settings. Fluorophores mentioned in staining are not automatically treated as acquired channels.

EVIDENCE OBJECT	REAGENTS / FLUOROPHORES PRESENT	CHANNELS ACTUALLY ACQUIRED
Western blot	532	Not deterministically stated in the acquisition block
Widefield	405/DAPI; 532; 555; 647	3; AF532-49014; 50 Cy 5; 49 DAPI; 515 - 545; 625 - 655; 335 - 383; 555 - 595
Widefield SIM — Apotome	405/DAPI; 532; 555; 647	3; AF532-49014; 50 Cy 5; 49 DAPI; 515 - 545; 625 - 655; 335 - 383; 555 - 595
Confocal	405/DAPI; 488; 532; 647	3; None; 488nm @0.5%; 639nm @0.2%; 405nm @0.2%; 528; 653; 353
Airyscan	405/DAPI; 488; 532; 647; 750	3; None; 488nm @1.50%; 639nm @0.1%; 405nm @0.2%; 528; 653; 353
SIM	405/DAPI; 488; 532; 647	2; SR HE LBF 405/488/561/642; 200 - 405, 200 - 488, 200 - 561, 200 - 642; 419 - 470, 503 - 546, 576 - 617, 657 - 800; T2-Axiocam 820 #2-SR; T1-Axiocam 820-SR; 488; 640
SIM²	405/DAPI; 488; 532; 647	2; SR HE LBF 405/488/561/642; 200 - 405, 200 - 488, 200 - 561, 200 - 642; 419 - 470, 503 - 546, 576 - 617, 657 - 800; T2-Axiocam 820 #2-SR; T1-Axiocam 820-SR; 488; 640

MODALITY ASSESSMENT / PART 1 OF 7

Western blot / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: Azure Sapphire FL. Objective: not explicitly stated. Platform: Azure Sapphire FL with Customizable Laser and Filter Modules, Enabling UV- NIR and Z-Plane(s). Structured source metadata values: 11.

VISIBLE OBSERVATION

A preserved presentation image is available for Western blot. BENNETT records the visible source presentation without creating a new native measurement.

SOURCE-REPORTED RESULT

The source identifies this object as Western blot, documents platform context as Azure Sapphire FL, and records detected dye/channel descriptors as 532.

LITERATURE CORRELATION

Consistent with peer-reviewed microscopy and antibody-validation guidance that presentation evidence must remain bounded by modality, controls and source documentation.

INTERPRETIVE LIMIT

Band position and displayed intensity do not independently establish analytical specificity, target identity, linearity, or treatment effect.

CLAIM CEILING

This tier supports a source-bounded statement that the historical Western blot evidence and documented settings are preserved for review; stronger biological or quantitative conclusions require compatible native data, controls and scientist review.

MODALITY ASSESSMENT / PART 2 OF 7

Widefield / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: ZEISS Axio Imager.M1. Objective: Plan-Apochromat 63x/1.40 Oil M27. Platform: Zeiss Axio Imager.M1 Widefield, using a Plan-Apochromat 63x/1.40 Oil M27, Axiocam 705, and X-Cite Xylis Lamp, was used with the following parameters for each dye:. Acquisition: Channels: 3; Scaling (Per Pixel): 0.110 µm X 0.110 µm; Image size (Pixels): 843 X 866; Image Size (Pixels): 843 X 866; Bit Depth: 14 Bit. Detector/camera: Imaging Device: Axiocam 705. Timing: Exposure Time: 200 ms; 150 ms. Illumination: Light source: X-Cite Xylis Lamp Intensity @20.00%; X-Cite Xylis Lamp Intensity @15.00%. Optical/scan: Effective NA: 1.4. Structured source metadata values: 39.

VISIBLE OBSERVATION

A preserved presentation image is available for Widefield. BENNETT records the visible source presentation without creating a new native measurement.

SOURCE-REPORTED RESULT

The source identifies this object as Widefield, documents platform context as ZEISS Axio Imager.M1, and records detected dye/channel descriptors as 405/DAPI; 532; 555; 647.

LITERATURE CORRELATION

Consistent with peer-reviewed microscopy and antibody-validation guidance that presentation evidence must remain bounded by modality, controls and source documentation.

INTERPRETIVE LIMIT

A presentation image supports gross localization and source-bounded co-occurrence, not direct binding, molecular colocalization, or native quantitative measurement.

CLAIM CEILING

This tier supports a source-bounded statement that the historical Widefield evidence and documented settings are preserved for review; stronger biological or quantitative conclusions require compatible native data, controls and scientist review.

MODALITY ASSESSMENT / PART 3 OF 7

Widefield SIM — Apotome / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: ZEISS Axio Observer.Z1/7 Apotome. Objective: EC Plan-Neofluar 63X/1.25 oil M27. Platform: Zeiss Axio Observer.Z1/7 Apotome SIM, using an EC Plan-Neofluar 63X/1.25 oil M27, Axiocam 807, and X-Cite Xylis Lamp was used with the following parameters for each dye:. Acquisition: Z-Stack: 87 Slices (8.6 µm); Channels: 3; Scaling (Per Pixel): 0.143 µm X 0.143 µm X 0.100 µm; Image size (Pixels): 450 X 428; Image Size (Pixels): 450 X 428; Bit Depth: 14 Bit. Detector/camera: Imaging Device: Axiocam 807 Mono. Timing: Exposure Time: 200 ms; 25 ms. Illumination: Light source: X-Cite Xylis Lamp Intensity @20.00%; X-Cite Xylis Lamp Intensity @10.00%. Optical/scan: Effective NA: 1.4; 1.25. Processing: Projection: View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); Background: Black. Structured source metadata values: 55.

VISIBLE OBSERVATION

A preserved presentation image is available for Widefield SIM — Apotome. BENNETT records the visible source presentation without creating a new native measurement.

SOURCE-REPORTED RESULT

The source identifies this object as Widefield SIM — Apotome, documents platform context as ZEISS Axio Observer.Z1/7 Apotome, and records detected dye/channel descriptors as 405/DAPI; 532; 555; 647.

LITERATURE CORRELATION

Consistent with peer-reviewed microscopy and antibody-validation guidance that presentation evidence must remain bounded by modality, controls and source documentation.

INTERPRETIVE LIMIT

Improved optical sectioning does not by itself establish reconstructed super-resolution or molecular interaction.

CLAIM CEILING

This tier supports a source-bounded statement that the historical Widefield SIM — Apotome evidence and documented settings are preserved for review; stronger biological or quantitative conclusions require compatible native data, controls and scientist review.

MODALITY ASSESSMENT / PART 4 OF 7

Confocal / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: ZEISS LSM 980. Objective: Plan-Apochromat 63X/1.40 oil DIC M27. Platform: Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:. Acquisition: Z-Stack: 181 Slices (4.8 µm); Channels: 3; Scaling (Per Pixel): 0.059 µm X 0.059 µm X 0.030 µm; Image size (Pixels): 397 X 371; Image Size (Pixels): 397 X 371; Bit Depth: 16 Bit. Detector/camera: Imaging Device: GaAsP-Pmt3; GaAsp-Pmt3; Detector Type: GaAsP-PMT; GaAsp-PMT. Timing: Pixel Time: 0.29µs; Frame Time: 4.97s. Illumination: Laser Wavelength: 488nm @0.5%; 639nm @0.2%. Optical/scan: Pinhole: 1.00AU / 53µm; 1.00AU / 66µm; Scan Zoom: 1.7; LSM Scan Speed: 11; Scan Direction: Bidirectional; Averaging: 8; Effective NA: 1.4. Processing: LSM Plus Mode: Filter: 7.3 (3D, Auto); Filter: 8.1 (3D, Auto). Structured source metadata values: 54.

VISIBLE OBSERVATION

A preserved presentation image is available for Confocal. BENNETT records the visible source presentation without creating a new native measurement.

SOURCE-REPORTED RESULT

The source identifies this object as Confocal, documents platform context as ZEISS LSM 980, and records detected dye/channel descriptors as 405/DAPI; 488; 532; 647.

LITERATURE CORRELATION

Consistent with peer-reviewed microscopy and antibody-validation guidance that presentation evidence must remain bounded by modality, controls and source documentation.

INTERPRETIVE LIMIT

Optical sectioning and source-bounded 3D presentation do not establish binding, mechanism, or population-level effect.

CLAIM CEILING

This tier supports a source-bounded statement that the historical Confocal evidence and documented settings are preserved for review; stronger biological or quantitative conclusions require compatible native data, controls and scientist review.

MODALITY ASSESSMENT / PART 5 OF 7

Airyscan / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: ZEISS LSM 980. Objective: Plan-Apochromat 63X/1.40 oil DIC M27. Platform: Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:. Acquisition: Z-Stack: 321 Slices (3.10 µm); Channels: 3; Scaling (Per Pixel): 35.30 nm X 35.30 nm X 10.00 nm; Image size (Pixels): 698 X 1033; 299 X 269; Image Size (Pixels): 698 X 1033; 299 X 269; Bit Depth: 16 Bit. Detector/camera: Imaging Device: Airyscan; Detector Type: GaAsP-PMT; GaAsp-PMT. Timing: Pixel Time: 1.10µs; Frame Time: 9.39s. Illumination: Laser Wavelength: 488nm @1.50%; 639nm @0.1%. Optical/scan: Pinhole: 5.00AU / 265µm; 5.00AU / 328µm; Scan Zoom: 2.0; LSM Scan Speed: 6; Scan Direction: Bidirectional; Averaging: 4; Effective NA: 1.4. Processing: Projection: View Angle 35°, Scale Z 10%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); Background: Black. Structured source metadata values: 59.

VISIBLE OBSERVATION

A preserved presentation image is available for Airyscan. BENNETT records the visible source presentation without creating a new native measurement.

SOURCE-REPORTED RESULT

The source identifies this object as Airyscan, documents platform context as ZEISS LSM 980, and records detected dye/channel descriptors as 405/DAPI; 488; 532; 647; 750.

LITERATURE CORRELATION

Consistent with peer-reviewed microscopy guidance that advanced-resolution outputs remain dependent on acquisition, reconstruction or localization quality control.

INTERPRETIVE LIMIT

Displayed feature separation remains reconstruction dependent; no unsupported numerical resolution or molecular architecture claim is permitted.

CLAIM CEILING

This tier supports a source-bounded statement that the historical Airyscan evidence and documented settings are preserved for review; stronger biological or quantitative conclusions require compatible native data, controls and scientist review.

MODALITY ASSESSMENT / PART 6 OF 7

SIM / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: ZEISS Elyra. Objective: Plan-Apochromat 63X/1.40 oil DIC M27. Platform: Zeiss Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1.25X Tubelens, was used with the following parameters for each dye:. Acquisition: Z-Stack: 55 Slices (1.62 µm); Channels: 2; Scaling (Per Pixel): 21.11 nm X 21.11 nm X 30.00 nm; Image size (Pixels): 1399 X 940; 262 X 222; Image Size (Pixels): 1399 X 940; 262 X 222; Bit Depth: 16 Bit. Detector/camera: Imaging Device: Axiocam 820 #2; Axiocam 820. Timing: Exposure Time: 150 ms; 120 ms; Frame Time: 1.59 s. Illumination: Laser Wavelength: 488 nm @2.0%; 640 nm @2.0%; Illumination Wavelength: 488; 640. Optical/scan: Scan Direction: Unidirectional; Effective NA: 1.4. Processing: Algorithm: SIM; Process Sampling: N/A; Result Sampling: 4; Projection: View Angle 35°, Scale Z 50%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); Background: Black. Structured source metadata values: 54.

VISIBLE OBSERVATION

A preserved presentation image is available for SIM. BENNETT records the visible source presentation without creating a new native measurement.

SOURCE-REPORTED RESULT

The source identifies this object as SIM, documents platform context as ZEISS Elyra, and records detected dye/channel descriptors as 405/DAPI; 488; 532; 647.

LITERATURE CORRELATION

Consistent with peer-reviewed microscopy guidance that advanced-resolution outputs remain dependent on acquisition, reconstruction or localization quality control.

INTERPRETIVE LIMIT

Reconstruction-dependent structural separation requires native reconstruction QC before numerical resolution or molecular dimensions can be claimed.

CLAIM CEILING

This tier supports a source-bounded statement that the historical SIM evidence and documented settings are preserved for review; stronger biological or quantitative conclusions require compatible native data, controls and scientist review.

MODALITY ASSESSMENT / PART 7 OF 7

SIM² / SOURCE EVIDENCE PRESERVED

DOCUMENTED SETTINGS

Platform: ZEISS Elyra. Objective: Plan-Apochromat 63X/1.40 oil DIC M27. Platform: Zeiss Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1.25X Tubelens, was used with the following parameters for each dye:. Acquisition: Z-Stack: 107 Slices (3.18 µm); Channels: 2; Scaling (Per Pixel): 21.11 nm X 21.11 nm X 30.00 nm; Image size (Pixels): 430 X 395; 181 X 181; Image Size (Pixels): 430 X 395; 181 X 181; Bit Depth: 16 Bit. Detector/camera: Imaging Device: Axiocam 820 #2; Axiocam 820. Timing: Exposure Time: 150 ms; 120 ms; Frame Time: 1.59 s. Illumination: Laser Wavelength: 488 nm @2.0%; 640 nm @2.0%; Illumination Wavelength: 488; 640. Optical/scan: Scan Direction: Unidirectional; Effective NA: 1.4. Processing: Algorithm: SIM2; Process Sampling: 4; Result Sampling: 4; Projection: View Angle 35°, Scale Z 50%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); View Angle 35°, Scale Z 10%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected); Background: Black. Structured source metadata values: 55.

VISIBLE OBSERVATION

A preserved presentation image is available for SIM². BENNETT records the visible source presentation without creating a new native measurement.

SOURCE-REPORTED RESULT

The source identifies this object as SIM², documents platform context as ZEISS Elyra, and records detected dye/channel descriptors as 405/DAPI; 488; 532; 647.

LITERATURE CORRELATION

Consistent with peer-reviewed microscopy guidance that advanced-resolution outputs remain dependent on acquisition, reconstruction or localization quality control.

INTERPRETIVE LIMIT

Further reconstruction-dependent segmentation does not establish molecular dimensions, structure identity, or mechanism without independent support.

CLAIM CEILING

This tier supports a source-bounded statement that the historical SIM² evidence and documented settings are preserved for review; stronger biological or quantitative conclusions require compatible native data, controls and scientist review.

CROSS-MODALITY SYNTHESIS

The preserved DC-000056 record contains the applicable NIR sequence: Western blot; Widefield; Widefield SIM — Apotome; Confocal; Airyscan; SIM; SIM². The historical boundary reflects the validated application available for the specific conjugate, deployed platform and workflow, not a universal scientific impossibility.

Different acquisitions are not treated as a quantitative intensity ladder. Any continuity statement remains qualitative and source bounded.

BENNETT LITERATURE CORRELATION — SOURCES INDEPENDENTLY VERIFIED

BENNETT uses independently verified primary scientific sources to test whether the interpretation is scientifically consistent and properly bounded. Literature correlation does not retroactively validate the Identifyn dataset or replace scientist review.

- Antibody and microscopy evidence require orthogonal controls and modality-appropriate interpretation; presentation images alone do not establish analytical specificity.

- Advanced-resolution presentation depends on acquisition, reconstruction or localization quality control before numerical resolution, molecular dimensions or cluster identity can be claimed.

REFERENCES

1. Uhlen M, et al. A proposal for validation of antibodies. Nat Methods. 2016;13:823-827. doi:10.1038/nmeth.3995.
2. Gustafsson MGL. Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. J Microsc. 2000;198:82-87. doi:10.1046/j.1365-2818.2000.00710.x.
3. Sage D, et al. Quantitative evaluation of software packages for single-molecule localization microscopy. Nat Methods. 2015;12:717-724. doi:10.1038/nmeth.3442.

RELEASE BOUNDARY

This assessment remains a draft pending scientist review. No publication is authorized. Any future native-file analysis must enter BENNETT as a new evidence snapshot with routed engine authority, native-data QA, methods, limitations, and an independently reviewed claim ceiling.

SOURCE RECONCILIATION / QA / PROVENANCE

SOURCE STATUS - PRESERVED / AUTO-RECONCILED

Historical Identifyn source material was subjected to BENNETT retrospective QA. Original source state and correction history remain preserved in provenance.

HIGH CONFIDENCE - AUTO-RECONCILED

Airyscan / Scaling (Per Pixel): 35.30 nm X 35.30 nm X 10.00 nm -> 0.03530 µm X 0.03530 µm X 0.01000 µm. Both numerical magnitude and unit were normalized, then checked against the reported field dimensions; no unit label was changed alone. Supporting evidence: Image Size (Pixels)=698 X 1033; Image Size (Scaled)=24.64 µm X 36.46 µm; PASS - INTERNALLY CONSISTENT. Documented sampling reproduces the reported physical field dimensions. Maximum field-dimension error 0.01%.

HIGH CONFIDENCE - AUTO-RECONCILED

SIM / Scaling (Per Pixel): 21.11 nm X 21.11 nm X 30.00 nm -> 0.02111 µm X 0.02111 µm X 0.03000 µm. Both numerical magnitude and unit were normalized, then checked against the reported field dimensions; no unit label was changed alone. Supporting evidence: Image Size (Pixels)=1399 X 940; Image Size (Scaled)=29.54 µm X 19.85 µm; PASS - INTERNALLY CONSISTENT. Documented sampling reproduces the reported physical field dimensions. Maximum field-dimension error 0.03%.

HIGH CONFIDENCE - AUTO-RECONCILED

SIM² / Scaling (Per Pixel): 21.11 nm X 21.11 nm X 30.00 nm -> 0.02111 µm X 0.02111 µm X 0.03000 µm. Both numerical magnitude and unit were normalized, then checked against the reported field dimensions; no unit label was changed alone. Supporting evidence: Image Size (Pixels)=430 X 395; Image Size (Scaled)=9.08 µm X 8.34 µm; PASS - INTERNALLY CONSISTENT. Documented sampling reproduces the reported physical field dimensions. Maximum field-dimension error 0.03%.

HIGH CONFIDENCE - SCIENTIST-AUTHORIZED CORRECTION

Cross-tier / treatment concentration: 200 µM; 2µM; 2 µM -> Etoposide = 2 µM. The governing scientist identified every 200 µM occurrence as a technician transcription error. The canonical historical concentration is 2 µM. Supporting evidence: Scientist-authorized correction supplied for the Identifyn historical archive; original technician text remains preserved unchanged. Authority: Dr. Brian T. Bennett. Preservation: Original source text and hashes remain unchanged; only the derivative canonical field is corrected.

UNRESOLVED - PRESERVED AS CONFLICT

Cross-tier / treatment duration: {"Western blot": ["20 hours"], "Widefield": ["24 hours"], "Widefield SIM — Apotome": ["24 hours"], "Confocal": ["24 hours"], "Airyscan": ["24 hours"], "SIM": ["24 hours"], "SIM²": ["24 hours"]} -> Preserved as modality-specific historical duration values. Genuinely different treatment durations are present across evidence stages, and no source or scientist correction resolves them. Supporting evidence: Only duration values from explicit etoposide-treatment sentences were classified; concentration, wavelength, resolution and pixel-size values were excluded.

BENNETT

AI-POWERED BIOLOGICAL INTELLIGENCE

BENNETT EVIDENCE REVIEW COMPLETE

INTERPRETATION TO EVIDENCE

BENNETT has completed the bounded retrospective assessment.
The following pages switch ownership to the preserved Identifyn record.

IDENTiFYN®

IDENTIFYN PRESERVED SOURCE EVIDENCE

ORIGINAL RECORD BEGINS

Images, source methods, acquisition settings and provenance follow in the locked Stepdown order. Scientific wording is preserved; missing modalities are not invented. No separate control record is present in the preserved product record.

PRODUCT-LEVEL STEPDOWN MAP

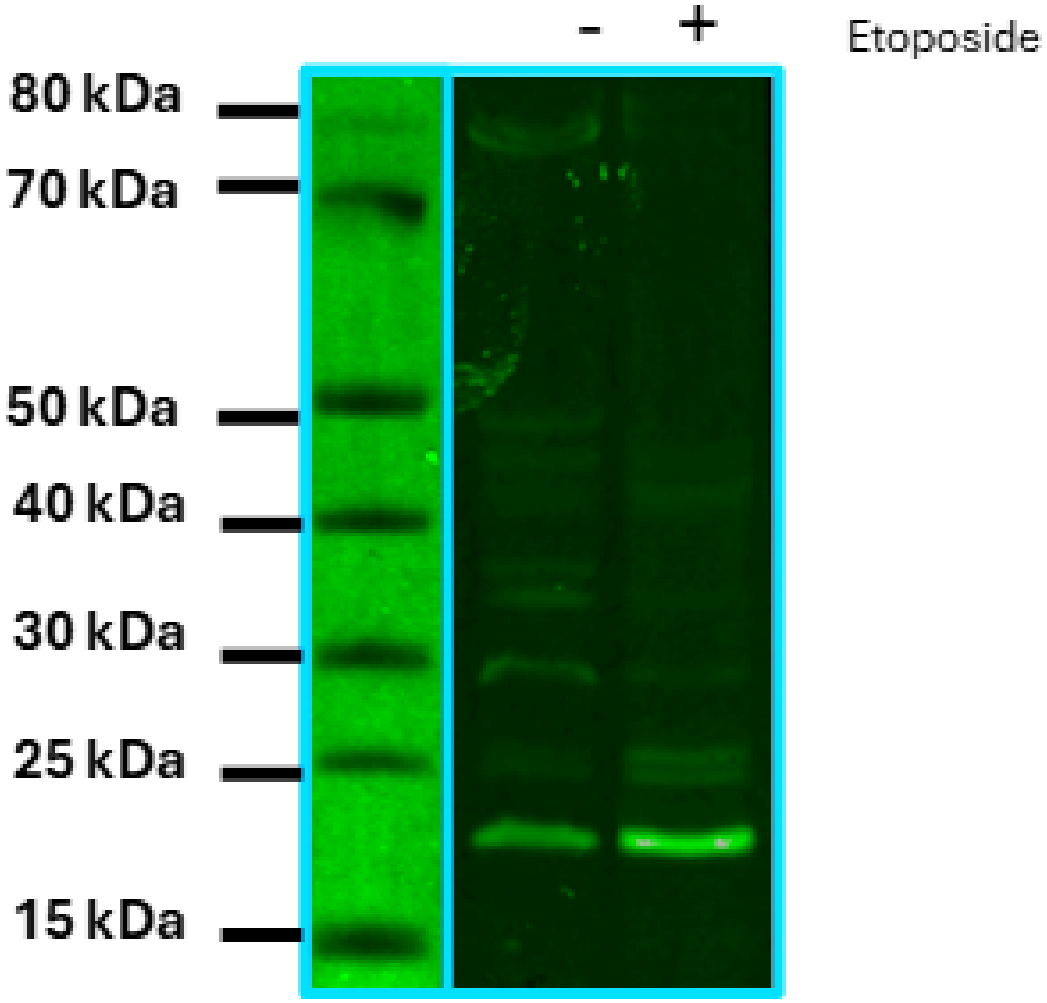
This dossier preserves the complete available evidence progression for DC-000056. Each tier remains tied to its original image, source method, instrument context and cryptographic identity. No separate control record is present in the preserved product record, and none is inferred.

CANONICAL STEPDOWN TIER	EVIDENCE STAGE	INSTRUMENT	STATUS
1	Western blot	Azure Sapphire FL	PRESERVED
2	Widefield	ZEISS Axio Imager.M1	PRESERVED
3	Widefield SIM - Apotome	ZEISS Axio Observer.Z1/7 Apotome	PRESERVED
4	Confocal	ZEISS LSM 980	PRESERVED
5	Airyscan	ZEISS LSM 980	PRESERVED
6	SIM	ZEISS Elyra	PRESERVED
7	SIM²	ZEISS Elyra	PRESERVED

INTERPRETIVE BOUNDARY

Ordering evidence by resolution tier does not make the modalities interchangeable. Each stage retains its acquisition environment and scientific limitations. This pilot organizes the record; it does not synthesize new biological claims.

ORIGINAL IMAGE EVIDENCE / WESTERN BLOT



EVIDENCE TIER	Western blot
INSTRUMENT	Azure Sapphire FL
CELL MODEL	HeLa
DETECTED DYES	532
METADATA VALUES	11

STRUCTURED ACQUISITION METADATA / WESTERN BLOT

The values below were deterministically extracted from the preserved Identifyn method PDF text. They remain source-documented metadata, not new BENNETT measurements.

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Azure Sapphire FL with Customizable Laser and Filter Modules, Enabling UV- NIR and Z-Plane(s)
Scanner	Azure Sapphire FL with Customizable Laser and Filter Modules, Enabling UV- NIR and Z-Plane(s)
Detection mode	Fluorescence
Laser	532nm
Emission Filter	572±28nm
Detector	PMT
Intensity	3
Pixel size	100 µm
Scan Speed	High
Quality	High
Focus	Membrane (0 µm in Z-axis)
Image	K. Small

SOURCE METHOD

The text below is carried from the preserved Identifyn source record. Typography and pagination have been normalized for this preservation dossier; scientific wording has not been editorially rewritten.

Bennett Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7)
Cat ID# - DC 000056

Expected MW: 15 kDa

HeLa cells were treated with 200 µM etoposide for 20 hours to induce DNA damage. Cells were then homogenized using an ultrasonication probe and RIPA lysis buffer, and then centrifuged to pellet the cell debris The lysate supernatant (100 µg) was mixed with NuPAGE™ LDS Sample Buffer (4X), heated at 80 °C for 10 min, and subjected to electrophoresis using NuPAGE™ 4 to 12% Bis-Tris, 1.0-1.5 mm, Mini Protein Gels in a Mini Gel Tank. The Thermo Fisher's PageRuler™ Prestained Protein Ladder, 10 to 180 kDa, was used as a molecular weight marker.

Post electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using the iBlot™ 3 Starter Kit PVDF. The transfer membrane was blocked with Blocker™ Casein for 1 hour, then washed 5 times with PBS. The blocked membrane was incubated with Mouse anti-Human H2AX Monoclonal - Identifyn® SRM Alexa Fluor™ 532 - Direct Conjugate for 2 hours, followed by five washes in 1X PBS. The membrane was dried for 10 minutes at room temperature before imaging.

Scanner = Azure Sapphire FL with Customizable Laser and Filter Modules, Enabling UV- NIR and Z-Plane(s)

Scan Settings

Detection mode = Fluorescence
Laser = 532nm
Emission Filter = 572±28nm
Detector = PMT
Intensity = 3
Pixel size = 100 µm
Scan Speed = High
Quality = High
Focus = Membrane (0 µm in Z-axis)

Post Processing

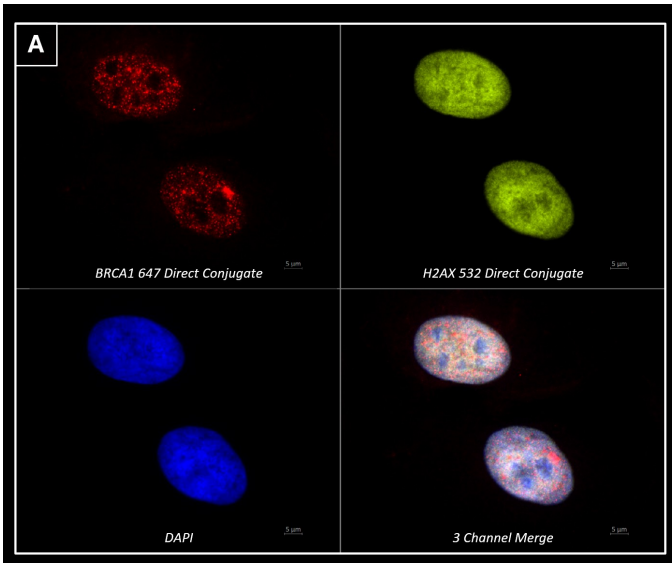
None

Image: K. Small

SOURCE PROVENANCE

Catalog	DC-000056
Stage	Western blot
Instrument	Azure Sapphire FL
Microscope configuration	Azure Sapphire FL with Customizable Laser and Filter Modules, Enabling UV- NIR and Z-Plane(s)
Structured source metadata values	11
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	7AC859441E879AA1559A7B8BEE8F6AF868A65919EB994AE71F249F37C7535603
Method PDF SHA-256	4C1769DBCF29C2DC0ACB35986446D162BB47C6CE2792D6DE68B48604296002DC

ORIGINAL IMAGE EVIDENCE / WIDEFIELD



B

Widefield

Reflector	Channel 1	Channel 2	Channel 3
Beam Splitter	50 Cy 5	AF532-49014	49 DAPI
Filter Ex. Wavelength	660	550	395
Filter Em. Wavelength	625 - 655	515 - 545	335 - 383
Contrast Method	665 - 715	555 - 595	420 - 470
Light Source	Fluorescence	Fluorescence	Fluorescence
Light Source Intensity	X-Cite Xylis Lamp	X-Cite Xylis Lamp	X-Cite Xylis Lamp
Channel Name	15.00 %	20.00 %	10.00 %
Channel Description	AF647	AF532	DAPI
Dye Name			
Channel Color	AF647	AF532	DAPI
Excitation Wavelength			
Emission Wavelength	653	528	353
Effective NA	668	553	465
Imaging Device	1.4	1.4	1.4
Camera Adapter	Axiocam 705	Axiocam 705	Axiocam 705
Exposure Time	1x Camera Adapter	1x Camera Adapter	1x Camera Adapter
Depth of Focus	150 ms	200 ms	25 ms
Binning Mode	1.03 µm	0.86 µm	0.72 µm
	2,2	2,2	2,2

Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 – (1H1D9/B7)
Cat ID# - DC 000056

Rabbit anti-Human BRCA1 Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647, IgG - (BLR023N)
Cat ID# - DC 000046

Baseline widefield imaging of the H2AX Alexa Fluor™ 532 Direct Conjugate, acquired following 2 µM etoposide treatment and co-imaged with the BRCA1 Alexa Fluor™ 647 Direct Conjugate, demonstrates robust nuclear localization and DNA damage–associated signal. These features are equivalently observed in the unconjugated H2AX condition (Cat. ID# PA-MM-000002), with Zeiss ZEN image acquisition settings displayed for transparency and held constant across conjugation states. Collectively, these data meet CDx go/no-go criteria for baseline equivalency and support downstream analytical and enrichment applications within Identifyn®'s step-down imaging methodology.

EVIDENCE TIER	Widefield
INSTRUMENT	ZEISS Axio Imager.M1
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 532; 555; 647
METADATA VALUES	39

STRUCTURED ACQUISITION METADATA / WIDEFIELD

The values below were deterministically extracted from the preserved Identifyn method PDF text. They remain source-documented metadata, not new BENNETT measurements.

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Zeiss Axio Imager.M1 Widefield, using a Plan-Apochromat 63x/1.40 Oil M27, Axiocam 705, and X-Cite Xylis Lamp, was used with the following parameters for each dye:
Objective	Plan-Apochromat 63x/1.40 Oil M27
Channels	3
Scaling (Per Pixel)	0.110 µm X 0.110 µm
Image size (Pixels)	843 X 866
Image Size (Scaled)	92.33 µm X 75.13 µm
Bit Depth	14 Bit
Image Center Position	X: 0.00 µm, Y: 0.00 µm
ROI Center Offset	X: 0.00 µm, Y: 0.00 µm
Reflector	AF532-49014 | 50 Cy 5 | 49 DAPI
Beam Splitter	550 | 660 | 395
Filter Excitation Wavelength	515 - 545 | 625 - 655 | 335 - 383
Filter Emission Wavelength	555 - 595 | 665 - 715 | 420 - 470
Contrast Method	Fluorescence
Light source	X-Cite Xylis Lamp Intensity @20.00% | X-Cite Xylis Lamp Intensity @15.00% | X-Cite Xylis Lamp Intensity @10.00%
Exposure Time	200 ms | 150 ms | 25 ms
Excitation Wavelength	528 | 653 | 353
Emission Wavelength	553 | 668 | 465
Effective NA	1.4
Imaging Device	Axiocam 705
Camera Adapter	1X
Depth of Focus	0.86 µm | 1.03 µm | 0.72 µm
Binning Mode	2,2

SOURCE METHOD

The text below is carried from the preserved Identifyn source record. Typography and pagination have been normalized for this preservation dossier; scientific wording has not been editorially rewritten.

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7)
Cat ID# - DC 000056

Rabbit anti-Human BRCA1 Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647, IgG - (BLR023N)
Cat ID# - DC 000046

H2AX, a variant of the histone protein H2A, and its primary function relates to the organization and structure of chromatin, as well as the cellular response to DNA damage. Here are the essential functions and roles of H2AX:

Nucleosome Formation: H2AX, like other histone proteins, contributes to the formation of nucleosomes. A nucleosome is the fundamental unit of chromatin, consisting of DNA wrapped around a core of histone proteins (including two molecules each of H2A, H2B, H3, and H4). This organization plays a crucial role in compacting DNA within the cell nucleus and regulating gene expression by controlling access to DNA.

DNA Damage Response (DDR): One of H2AX's key functions, distinct from those of other histone proteins, is its function in the DNA damage response. When DNA suffers double-strand breaks (DSBs), H2AX is phosphorylated at a specific serine residue (serine 139), leading to the formation of γ-H2AX (gamma H2AX). This phosphorylation serves as an early signal of DNA damage.

Recruitment of DNA Repair Proteins: Phosphorylation of H2AX to form γ-H2AX creates a binding site for various proteins involved in DNA repair. It helps recruit proteins involved in the recognition, signaling, and repair of DNA damage, such as MDC1 (Mediator of DNA Damage Checkpoint 1), 53BP1 (p53-binding protein 1), BRCA1 (Breast Cancer 1), and others. These proteins work together to repair the damage and maintain genomic stability.

Chromatin Remodeling: Through its role in signaling and repair, H2AX plays a role in chromatin remodeling during the DNA damage response. The formation of γ-H2AX marks regions where chromatin structure is altered to allow access for repair proteins and facilitate the repair process.

H2AX's functions are fascinating studies in molecular biology. They involve a complex interplay of structural and signaling roles. Its structural role in chromatin and its specialized role in signaling and facilitating the DNA damage response, particularly in double-strand breaks, are of immense interest. Through its phosphorylation to form γ-H2AX, it acts as a key marker for DNA damage and plays an essential role in the recruitment and organization of proteins implicated in the DNA repair process.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2μM Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.

The second primary antibody, Rabbit anti-Human BRCA1 Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647, IgG - (BLR023N) was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI (cat# P36962).

Control Data - Primary Unconjugated Antibody

Microscope

Zeiss Axio Imager.M1 Widefield, using a Plan-Apochromat 63x/1.40 Oil M27, Axiocam 705, and X-Cite Xylis Lamp, was used with the following parameters for each dye:

Single Plane (2D)

Channels = 3
Scaling (Per Pixel) = 0.110 µm X 0.110 µm
Image size (Pixels) = 843 X 866
Image Size (Scaled) = 92.33 µm X 75.13 µm
Bit Depth = 14 Bit
Image Center Position = X: 0.00 µm, Y: 0.00 µm
ROI Center Offset = X: 0.00 µm, Y: 0.00 µm

532-

Reflector = AF532-49014
Beam Splitter = 550
Filter Excitation Wavelength = 515 - 545
Filter Emission Wavelength = 555 - 595
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @20.00%
Exposure Time = 200 ms
Excitation Wavelength = 528
Emission Wavelength = 553
Effective NA = 1.4
Imaging Device = Axiocam 705
Camera Adapter = 1X
Depth of Focus = 0.86 µm
Binning Mode = 2,2

647 -

Reflector = 50 Cy 5
Beam Splitter = 660
Filter Excitation Wavelength = 625 - 655
Filter Emission Wavelength = 665 - 715
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @15.00%
Exposure Time = 150 ms
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.4
Imaging Device = Axiocam 705
Camera Adapter = 1X
Depth of Focus = 1.03 µm
Binning Mode = 2,2

DAPI -

Reflector = 49 DAPI
Beam Splitter = 395
Filter Excitation Wavelength = 335 - 383
Filter Emission Wavelength = 420 - 470
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @10.00%
Exposure Time = 25 ms
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Imaging Device = Axiocam 705
Camera Adapter = 1X
Depth of Focus = 0.72 µm
Binning Mode = 2,2

Split View - Panel A

The top-left panel shows Rabbit anti-Human BRCA1 Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647, IgG - (BLR023N). The top-right panel shows Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7). The lower-left panel shows DAPI staining, and the bottom-right panel shows the three-channel merged image.

Zen Acquisition Info Tab - Panel B

Shown is the acquisition of the 532 nm channel (H2AX Alexa Fluor™ 532 Direct Conjugate) using an X-Cite Xylis illumination source operated at 20% output and detected with an Axiocam 705 camera using a 200 ms exposure time. Additionally shown is the 647 channel (BRCA1 Alexa Fluor™ 647 Direct Conjugate) using 15% input with a 150ms exposure time.

Summary

Baseline widefield imaging of the H2AX Alexa Fluor™ 532 Direct Conjugate, acquired following 2 µM etoposide treatment and co-imaged with the BRCA1 Alexa Fluor™ 647 Direct Conjugate, demonstrates robust nuclear localization and DNA damage-associated signal. These features are equivalently observed in the unconjugated H2AX condition (Cat. ID# PA-MM-000002), with Zeiss ZEN image acquisition settings displayed for transparency and held constant across conjugation states. Collectively, these data meet CDx go/no-go criteria for baseline equivalency and support downstream analytical and enrichment applications within Identifyn®'s step-down imaging methodology.

Image Corrections

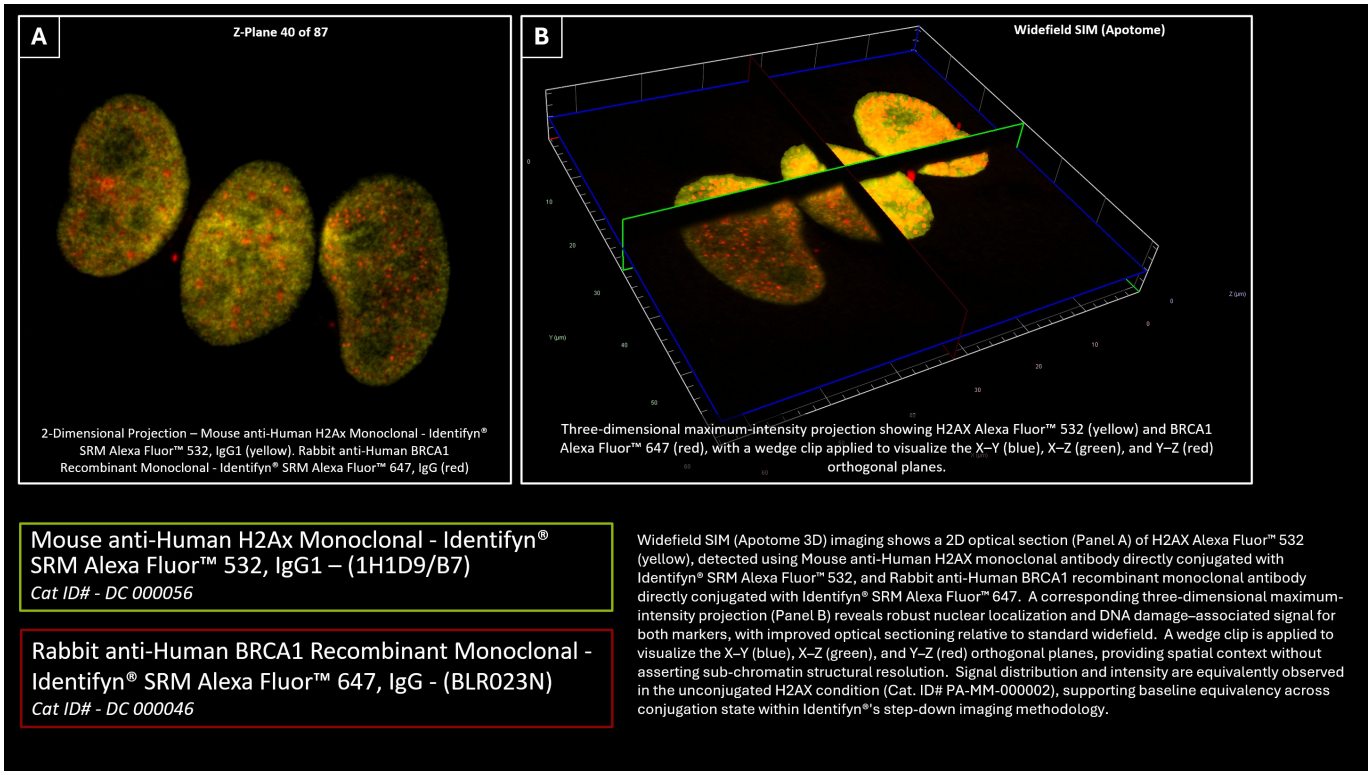
NONE

Image by E.F.Simon
Image Analysis and Data Presentation by B.T. Bennett

SOURCE PROVENANCE

Catalog	DC-000056
Stage	Widefield
Instrument	ZEISS Axio Imager.M1
Microscope configuration	Zeiss Axio Imager.M1 Widefield, using a Plan-Apochromat 63x/1.40 Oil M27, Axiocam 705, and X-Cite Xylis Lamp, was used with the following parameters for each dye:
Structured source metadata values	39
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	2CA4BB335B9A357AED731231F97B140471AE5AE9F782397A76DF842F735670A5
Method PDF SHA-256	63C519382897BE06BA6BABCB66E56068CED7342A331C3D2F15DF3CE4F8B8C500

ORIGINAL IMAGE EVIDENCE / WIDEFIELD SIM - APOTOME



2-Dimensional Projection – Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 (yellow). Rabbit anti-Human BRCA1 Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647, IgG (red)

Three-dimensional maximum-intensity projection showing H2AX Alexa Fluor™ 532 (yellow) and BRCA1 Alexa Fluor™ 647 (red), with a wedge clip applied to visualize the X–Y (blue), X–Z (green), and Y–Z (red) orthogonal planes.

Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 – (1H1D9/B7)
Cat ID# - DC 000056

Rabbit anti-Human BRCA1 Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647, IgG - (BLR023N)
Cat ID# - DC 000046

Widefield SIM (Apotome 3D) imaging shows a 2D optical section (Panel A) of H2AX Alexa Fluor™ 532 (yellow), detected using Mouse anti-Human H2AX monoclonal antibody directly conjugated with Identifyn® SRM Alexa Fluor™ 532, and Rabbit anti-Human BRCA1 recombinant monoclonal antibody directly conjugated with Identifyn® SRM Alexa Fluor™ 647. A corresponding three-dimensional maximum-intensity projection (Panel B) reveals robust nuclear localization and DNA damage–associated signal for both markers, with improved optical sectioning relative to standard widefield. A wedge clip is applied to visualize the X–Y (blue), X–Z (green), and Y–Z (red) orthogonal planes, providing spatial context without asserting sub-chromatin structural resolution. Signal distribution and intensity are equivalently observed in the unconjugated H2AX condition (Cat. ID# PA-MM-000002), supporting baseline equivalency across conjugation state within Identifyn®'s step-down imaging methodology.

EVIDENCE TIER	Widefield SIM - Apotome
INSTRUMENT	ZEISS Axio Observer.Z1/7 Apotome
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 532; 555; 647
METADATA VALUES	55

STRUCTURED ACQUISITION METADATA / WIDEFIELD SIM - APOTOME

The values below were deterministically extracted from the preserved Identifyn method PDF text. They remain source-documented metadata, not new BENNETT measurements.

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Zeiss Axio Observer.Z1/7 Apotome SIM, using an EC Plan-Neofluar 63X/1.25 oil M27, Axiocam 807, and X-Cite Xylis Lamp was used with the following parameters for each dye:
Objective	EC Plan-Neofluar 63X/1.25 oil M27
Channels	3
Scaling (Per Pixel)	0.143 µm X 0.143 µm X 0.100 µm
Image size (Pixels)	450 X 428
Image Size (Scaled)	64.29 µm X 61.14 µm
Bit Depth	14 Bit
Image Center Position	X: 53.96 µm, Y: 47.57 µm
Stage Position	X: 53.96 µm, Y: 47.57 µm
ROI Center Offset	X: 1.14 µm, Y: 0.00 µm
Z-Stack	87 Slices (8.6 µm)
Reflector	AF532-49014 | 50 Cy 5 | 49 DAPI
Beam Splitter	550 | 660 | 395
Filter Excitation Wavelength	515 - 545 | 625 - 655 | 335 - 383
Filter Emission Wavelength	555 - 595 | 665 - 715 | 420 - 470
Contrast Method	Fluorescence
Light source	X-Cite Xylis Lamp Intensity @20.00% | X-Cite Xylis Lamp Intensity @10.00%
Exposure Time	200 ms | 25 ms
Excitation Wavelength	528 | 653 | 353
Emission Wavelength	553 | 668 | 465
Effective NA	1.4 | 1.25
Imaging Device	Axiocam 807 Mono
Camera Adapter	1X
Depth of Focus	1.07 µm | 1.30 µm | 0.90 µm
Binning Mode	2,2
Apotome SIM Image Processing	Display Mod" "Optical Sectioning" - enabled correction selected.
3D Maximum Projection	Precise
Appearance	Threshold 13% all channels, Ramp 0% all channels, Maximum 100% all channels
Background	Black
Light	Brightness 100%, Enabled Tone Mapping
Projection	View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)
X-Y	Activate was selected, textured opaque was chosen, and a blue outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

FIELD	SOURCE-DOCUMENTED VALUE
Position of Clip	-9% | 0%
Clip X	0°
Clip Z	0°
X-Z	Activate was selected, textured opaque was chosen, and a green outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.
Clip Y	0° | -0°
Y-Z	Activate was selected, textured opaque was chosen, and a red outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

SOURCE METHOD

The text below is carried from the preserved Identifyn source record. Typography and pagination have been normalized for this preservation dossier; scientific wording has not been editorially rewritten.

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7)
Cat ID# - DC 000056

Rabbit anti-Human BRCA1 Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647, IgG - (BLR023N)
Cat ID# - DC 000046

H2AX, a variant of the histone protein H2A, and its primary function relates to the organization and structure of chromatin, as well as the cellular response to DNA damage. Here are the essential functions and roles of H2AX:

Nucleosome Formation: H2AX, like other histone proteins, contributes to the formation of nucleosomes. A nucleosome is the fundamental unit of chromatin, consisting of DNA wrapped around a core of histone proteins (including two molecules each of H2A, H2B, H3, and H4). This organization plays a crucial role in compacting DNA within the cell nucleus and regulating gene expression by controlling access to DNA.

DNA Damage Response (DDR): One of H2AX's key functions, distinct from those of other histone proteins, is its function in the DNA damage response. When DNA suffers double-strand breaks (DSBs), H2AX is phosphorylated at a specific serine residue (serine 139), leading to the formation of γ-H2AX (gamma H2AX). This phosphorylation serves as an early signal of DNA damage.

Recruitment of DNA Repair Proteins: Phosphorylation of H2AX to form γ-H2AX creates a binding site for various proteins involved in DNA repair. It helps recruit proteins involved in the recognition, signaling, and repair of DNA damage, such as MDC1 (Mediator of DNA Damage Checkpoint 1), 53BP1 (p53-binding protein 1), BRCA1 (Breast Cancer 1), and others. These proteins work together to repair the damage and maintain genomic stability.

Chromatin Remodeling: Through its role in signaling and repair, H2AX plays a role in chromatin remodeling during the DNA damage response. The formation of γ-H2AX marks regions where chromatin structure is altered to allow access for repair proteins and facilitate the repair process.

H2AX's functions are fascinating studies in molecular biology. They involve a complex interplay of structural and signaling roles. Its structural role in chromatin and its specialized role in signaling and facilitating the DNA damage response, particularly in double-strand breaks, are of immense interest. Through its phosphorylation to form γ-H2AX, it acts as a key marker for DNA damage and plays an essential role in the recruitment and organization of proteins implicated in the DNA repair process.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2μM Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.

The second primary antibody, Rabbit anti-Human BRCA1 Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647, IgG - (BLR023N) was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI (cat# P36962).

Control Data - Primary Unconjugated Antibody

Microscope

Zeiss Axio Observer.Z1/7 Apotome SIM, using an EC Plan-Neofluar 63X/1.25 oil M27, Axiocam 807, and X-Cite Xylis Lamp was used with the following parameters for each dye:

Single Plane (2D) - Panel A, Z-plane 40 of 87

Channels = 3
Scaling (Per Pixel) = 0.143 µm X 0.143 µm X 0.100 µm
Image size (Pixels) = 450 X 428
Image Size (Scaled) = 64.29 µm X 61.14 µm
Bit Depth = 14 Bit
Image Center Position = X: 53.96 µm, Y: 47.57 µm
Stage Position = X: 53.96 µm, Y: 47.57 µm
ROI Center Offset = X: 1.14 µm, Y: 0.00 µm

Z Stack - (3D) - Panel B

Z-Stack = 87 Slices (8.6 µm)

Channels = 3
Scaling (Per Pixel) = 0.143 µm X 0.143 µm X 0.100 µm
Image size (Pixels) = 450 X 428
Image Size (Scaled) = 64.29 µm X 61.14 µm
Bit Depth = 14 Bit
Image Center Position = X: 53.96 µm, Y: 47.57 µm
Stage Position = X: 53.96 µm, Y: 47.57 µm
ROI Center Offset = X: 1.14 µm, Y: 0.00 µm

532 -

Reflector = AF532-49014
Beam Splitter = 550
Filter Excitation Wavelength = 515 - 545
Filter Emission Wavelength = 555 - 595
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @20.00%
Exposure Time = 200 ms
Excitation Wavelength = 528
Emission Wavelength = 553
Effective NA = 1.4
Imaging Device = Axiocam 807 Mono
Camera Adapter = 1X
Depth of Focus = 1.07 µm
Binning Mode = 2,2

647 -

Reflector = 50 Cy 5
Beam Splitter = 660
Filter Excitation Wavelength = 625 - 655
Filter Emission Wavelength = 665 - 715
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @20.00%
Exposure Time = 200 ms
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.25
Imaging Device = Axiocam 807 Mono
Camera Adapter = 1X
Depth of Focus = 1.30 µm
Binning Mode = 2,2

DAPI - (not shown)

Reflector = 49 DAPI
Beam Splitter = 395
Filter Excitation Wavelength = 335 - 383
Filter Emission Wavelength = 420 - 470
Contrast Method = Fluorescence
Light source = X-Cite Xylis Lamp Intensity @10.00%
Exposure Time = 25 ms
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Imaging Device = Axiocam 807 Mono
Camera Adapter = 1X
Depth of Focus = 0.90 µm
Binning Mode = 2,2

Post Processing - SIM Processing

Apotome SIM Image Processing = Display Mod" "Optical Sectioning" - enabled correction selected. Normalization, Phase Corrector, Fourier Filter, and Deconvolution were NOT used.

2-Dimensional View - Panel A

Shows Rabbit anti-Human BRCA1 Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647, IgG - (BLR023N) and Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7) at z-plane 40 of 87.

3D Image Processing - Panel B

3D Maximum Projection = Precise
Appearance = Threshold 13% all channels, Ramp 0% all channels, Maximum 100% all channels
Background = Black
Light = Brightness 100%, Enabled Tone Mapping
Projection = View Angle 35°, Scale Z 100%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Clipping Image Process - Panel B

Clipping planes are introduced in Panel B to highlight the H2AX/BRCA1 within the nuclear compartment by adjusting or cutting away portions of the image using a triple-wedge cut, removing parts of the image in the X-Y, X-Z, and Y-Z planes.

X-Y = Activate was selected, textured opaque was chosen, and a blue outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

Position of Clip = -9%
Clip X = 0°
Clip Z = 0°

X-Z = Activate was selected, textured opaque was chosen, and a green outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

Position of Clip =0%
Clip Y = 0°
Clip Z = 0°

Y-Z = Activate was selected, textured opaque was chosen, and a red outline of the clipped perimeter was shown; Clip Back was selected, and Clip Front was NOT selected.

Position of Clip = 0%
Clip Y = -0°
Clip Z = 0°

Panel B shows a wedge-cut view displayed from the top with a slight backward rotation, highlighting the region of wedge removal and revealing the full depth of signal across the z-stack. This orientation provides an alternative view of wedge geometry and confirms intranuclear localization of the H2AX/532, as indicated by colocalization with BRCA1/647.

Summary

Widefield SIM (Apotome 3D) imaging shows a 2D optical section (Panel A) of H2AX Alexa Fluor™ 532 (yellow), detected using Mouse anti-Human H2AX monoclonal antibody directly conjugated with Identifyn® SRM Alexa Fluor™ 532, and Rabbit anti-Human BRCA1 recombinant monoclonal antibody directly conjugated with Identifyn® SRM Alexa Fluor™ 647. A corresponding three-dimensional maximum-intensity projection (Panel B) reveals robust nuclear localization and DNA damage-associated signal for both markers, with improved optical sectioning relative to standard widefield. A wedge clip is applied to visualize the X-Y (blue), X-Z (green), and Y-Z (red) orthogonal planes, providing spatial context without asserting sub-chromatin structural resolution. Signal distribution and intensity are similarly observed in the unconjugated H2AX condition (Cat. ID# PA-MM-000002), supporting baseline equivalence across conjugation states. These data meet CDx go/no-go criteria for conjugation equivalency, analytical sensitivity, and localization specificity, and support advancement of this marker for DNA damage enrichment and downstream stratification workflows within Identifyn®'s step-down imaging methodology.

Image Corrections

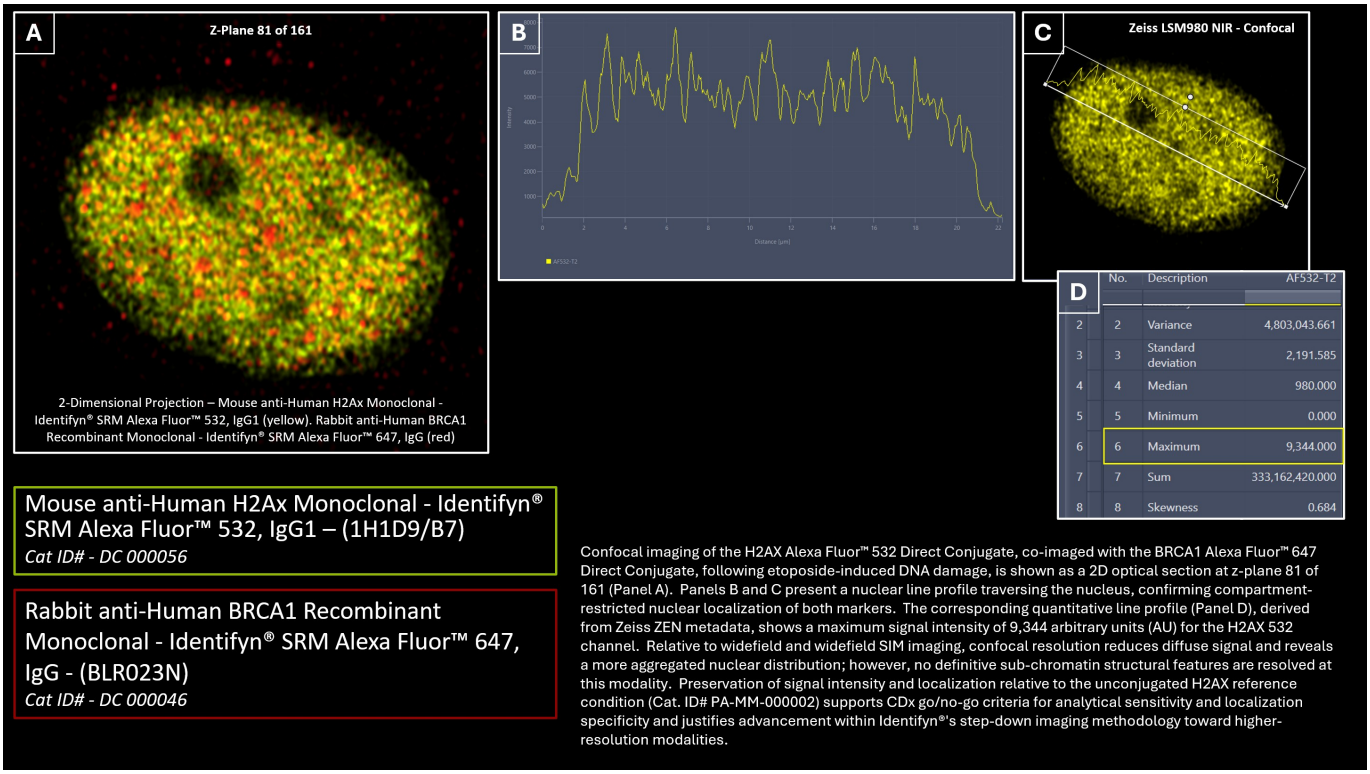
NONE

Image by E.F.Simon
Image Analysis and Data Presentation by B.T. Bennett

SOURCE PROVENANCE

Catalog	DC-000056
Stage	Widefield SIM - Apotome
Instrument	ZEISS Axio Observer.Z1/7 Apotome
Microscope configuration	Zeiss Axio Observer.Z1/7 Apotome SIM, using an EC Plan-Neofluar 63X/1.25 oil M27, Axiocam 807, and X-Cite Xylis Lamp was used with the following parameters for each dye:
Structured source metadata values	55
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	1B92B41ECB4B45358D903C5F349426C80A5D3577C629E4D3F7CA810C49F44EDA
Method PDF SHA-256	393D698EE23623B3B94E0A5BB4CF6F9E05F6D6669ED1DAA799E43B3428C750FD

ORIGINAL IMAGE EVIDENCE / CONFOCAL



EVIDENCE TIER	Confocal
INSTRUMENT	ZEISS LSM 980
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 532; 647
METADATA VALUES	54

STRUCTURED ACQUISITION METADATA / CONFOCAL

The values below were deterministically extracted from the preserved Identifyn method PDF text. They remain source-documented metadata, not new BENNETT measurements.

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:
Objective	Plan-Apochromat 63X/1.40 oil DIC M27
Z-Stack	181 Slices (4.8 µm)
Channels	3
Scaling (Per Pixel)	0.059 µm X 0.059 µm X 0.030 µm
Image size (Pixels)	397 X 371
Image Size (Scaled)	23.35 µm X 21.82 µm
Bit Depth	16 Bit
Image Center Position	X: 59.35 µm, Y: 53.08 µm
Stage Position	X: 59.35 µm, Y: 53.08 µm
ROI Center Offset	X: 1.14 µm, Y: 0.00 µm
Reflector	None
Contrast Method	Fluorescence
Pinhole	1.00AU / 53µm | 1.00AU / 66µm | 1.00AU / 43µm
LMS MBS	MBS 488/639 & 405/730
LMS SBS	Mirror | SBS SP 615
Laser Wavelength	488nm @0.5% | 639nm @0.2% | 405nm @0.2%
Laser Blanking	Enabled
Scan Mode	Frame
Scan Zoom	1.7
Rotation	0°
Sampling	1.20
Pixel Time	0.29µs
Frame Time	4.97s
LSM Scan Speed	11
Scan Direction	Bidirectional
Line Step	1
Averaging	8
Excitation Wavelength	528 | 653 | 353
Emission Wavelength	553 | 668 | 465
Effective NA	1.4
Detection Wavelength	530 - 580 | 643 - 740 | 440 - 465
Imaging Device	GaAsP-Pmt3 | GaAsp-Pmt3

FIELD	SOURCE-DOCUMENTED VALUE
Detector Type	GaAsP-PMT | GaAsp-PMT
Detector Gain	550 V | 500 V
Detector Offset	0
Detector Digital Gain	1.0
LSM Plus Mode	Filter: 7.3 (3D, Auto) | Filter: 8.1 (3D, Auto) | Filter: 6.7 (3D, Auto)
Profile Definition	Stroke Thickness 1.5, Normal View, Grid Distance 1
Profile View	X and Y axes were set to Auto - X (Min 0.0 and Max 22.2), Y (Min 149 and Max 8184)

SOURCE METHOD

The text below is carried from the preserved Identifyn source record. Typography and pagination have been normalized for this preservation dossier; scientific wording has not been editorially rewritten.

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7)
Cat ID# - DC 000056

Rabbit anti-Human BRCA1 Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647, IgG - (BLR023N)
Cat ID# - DC 000046

H2AX, a variant of the histone protein H2A, and its primary function relates to the organization and structure of chromatin, as well as the cellular response to DNA damage. Here are the essential functions and roles of H2AX:

Nucleosome Formation: H2AX, like other histone proteins, contributes to the formation of nucleosomes. A nucleosome is the fundamental unit of chromatin, consisting of DNA wrapped around a core of histone proteins (including two molecules each of H2A, H2B, H3, and H4). This organization plays a crucial role in compacting DNA within the cell nucleus and regulating gene expression by controlling access to DNA.

DNA Damage Response (DDR): One of H2AX's key functions, distinct from those of other histone proteins, is its function in the DNA damage response. When DNA suffers double-strand breaks (DSBs), H2AX is phosphorylated at a specific serine residue (serine 139), leading to the formation of γ-H2AX (gamma H2AX). This phosphorylation serves as an early signal of DNA damage.

Recruitment of DNA Repair Proteins: Phosphorylation of H2AX to form γ-H2AX creates a binding site for various proteins involved in DNA repair. It helps recruit proteins involved in the recognition, signaling, and repair of DNA damage, such as MDC1 (Mediator of DNA Damage Checkpoint 1), 53BP1 (p53-binding protein 1), BRCA1 (Breast Cancer 1), and others. These proteins work together to repair the damage and maintain genomic stability.

Chromatin Remodeling: Through its role in signaling and repair, H2AX plays a role in chromatin remodeling during the DNA damage response. The formation of γ-H2AX marks regions where chromatin structure is altered to allow access for repair proteins and facilitate the repair process.

H2AX's functions are fascinating studies in molecular biology. They involve a complex interplay of structural and signaling roles. Its structural role in chromatin and its specialized role in signaling and facilitating the DNA damage response, particularly in double-strand breaks, are of immense interest. Through its phosphorylation to form γ-H2AX, it acts as a key marker for DNA damage and plays an essential role in the recruitment and organization of proteins implicated in the DNA repair process.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2μM Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.

The second primary antibody, Rabbit anti-Human BRCA1 Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647, IgG - (BLR023N) was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI (cat# P36962).

Control Data - Primary Unconjugated Antibody

Microscope

Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:

Z Stack - (3D) - Panels A, B, C, and D, at z-plane 81 of 161

Z-Stack = 181 Slices (4.8 µm)

Channels = 3
Scaling (Per Pixel) = 0.059 µm X 0.059 µm X 0.030 µm
Image size (Pixels) = 397 X 371
Image Size (Scaled) = 23.35 µm X 21.82 µm
Bit Depth = 16 Bit
Image Center Position = X: 59.35 µm, Y: 53.08 µm
Stage Position = X: 59.35 µm, Y: 53.08 µm
ROI Center Offset = X: 1.14 µm, Y: 0.00 µm

532 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00AU / 53µm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = Mirror
Laser Wavelength = 488nm @0.5%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.7
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.29µs
Frame Time = 4.97s
LSM Scan Speed = 11
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 528
Emission Wavelength = 553
Effective NA = 1.4
Detection Wavelength = 530 - 580
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 550 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 7.3 (3D, Auto)

647 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00AU / 66µm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = SBS SP 615
Laser Wavelength = 639nm @0.2%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.7
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.29µs
Frame Time = 4.97s
LSM Scan Speed = 11
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.4
Detection Wavelength = 643 - 740
Imaging Device = GaAsP-Pmt3
Detector Type = GaAsP-PMT
Detector Gain = 500 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 8.1 (3D, Auto)

DAPI - (Not Shown)

Reflector = None
Contrast Method = Fluorescence
Pinhole = 1.00AU / 43µm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = Mirror
Laser Wavelength = 405nm @0.2%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 1.7
Rotation = 0°
Sampling = 1.20
Pixel Time = 0.29µs
Frame Time = 4.97s
LSM Scan Speed = 11
Scan Direction = Bidirectional
Line Step = 1
Averaging = 8
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Detection Wavelength = 440 - 465
Imaging Device = GaAsp-Pmt3
Detector Type = GaAsp-PMT
Detector Gain = 500 V
Detector Offset = 0
Detector Digital Gain = 1.0
LSM Plus Mode = Filter: 6.7 (3D, Auto)

2-Dimensional View - Panel A

Shows Rabbit anti-Human BRCA1 Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647, IgG - (BLR023N) and Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7) at z-plane 81 of 161.

Profile View Display - Panels B & C

Profile Definition = Stroke Thickness 1.5, Normal View, Grid Distance 1
Profile View = X and Y axes were set to Auto - X (Min 0.0 and Max 22.2), Y (Min 149 and Max 8184)

Measured at Z-plane 81 of 161

Histogram View - Panel D, Used to Show Max Signal for H2AX/532 Direct Conjugate

Histogram Definition - Normal, Histo Table was Frequency, Bin Count 256, Bin Size 256, Lower Threshold 0, Upper Threshold 65535, Showing Threshold, and deploying Logarithmic Binning

Histogram View - X and Y axis auto, Channel Transparency 100%, Statistic Table, Frequency Table, Internal Measurements, and Image were selected.

Summary

Confocal imaging of the H2AX Alexa Fluor™ 532 Direct Conjugate, co-imaged with the BRCA1 Alexa Fluor™ 647 Direct Conjugate, following etoposide-induced DNA damage, is shown as a 2D optical section at z-plane 81 of 161 (Panel A). Panels B and C present a nuclear line profile traversing the nucleus, confirming compartment-restricted nuclear localization of both markers. The corresponding quantitative line profile (Panel D), derived from Zeiss ZEN metadata, shows a maximum signal intensity of 9,344 arbitrary units (AU) for the H2AX 532 channel. Relative to widefield and widefield SIM imaging, confocal resolution reduces diffuse signal and reveals a more aggregated nuclear distribution; however, no definitive sub-chromatin structural features are resolved at this modality. Preservation of signal intensity and localization relative to the unconjugated H2AX reference condition (Cat. ID# PA-MM-000002) supports CDx go/no-go criteria for analytical sensitivity and localization specificity and justifies advancement within Identifyn®'s step-down imaging methodology toward higher-resolution modalities.

Image Corrections

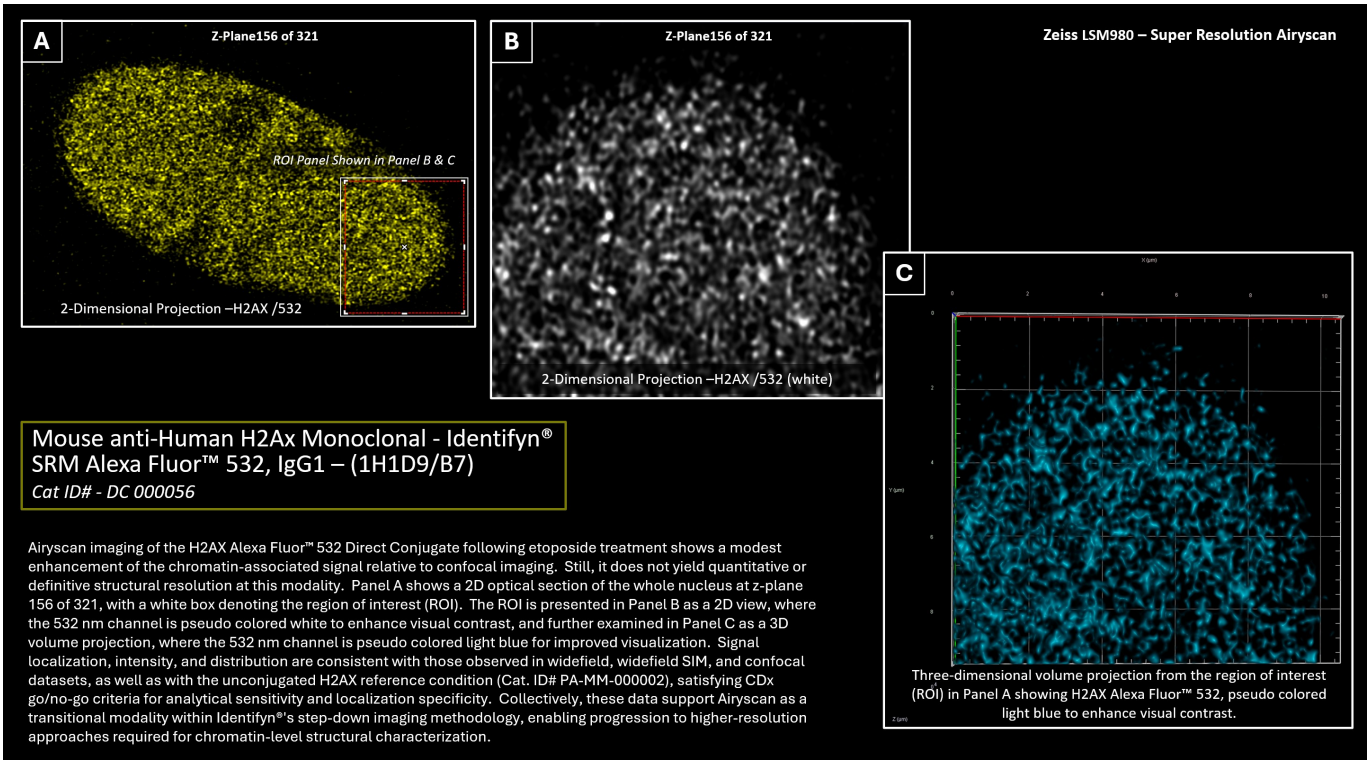
NONE

Image by J.K. Bennett
Image Analysis and Data Presentation by B.T. Bennett

SOURCE PROVENANCE

Catalog	DC-000056
Stage	Confocal
Instrument	ZEISS LSM 980
Microscope configuration	Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:
Structured source metadata values	54
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	182BD541A91C65815F2A68B803C87DD5F672D4958D5EC47E0C9B61061F1C5BDE
Method PDF SHA-256	ADB5CAA4A8913DA83D5AEC5173E9DA55E4E11CE7DE3919A3DC766883D3111DB7

ORIGINAL IMAGE EVIDENCE / AIRYSCAN



Mouse anti-Human H2Ax Monoclonal - Identifyn®
SRM Alexa Fluor™ 532, IgG1 – (1H1D9/B7)
Cat ID# - DC 000056

Airyscan imaging of the H2AX Alexa Fluor™ 532 Direct Conjugate following etoposide treatment shows a modest enhancement of the chromatin-associated signal relative to confocal imaging. Still, it does not yield quantitative or definitive structural resolution at this modality. Panel A shows a 2D optical section of the whole nucleus at z-plane 156 of 321, with a white box denoting the region of interest (ROI). The ROI is presented in Panel B as a 2D view, where the 532 nm channel is pseudo colored white to enhance visual contrast, and further examined in Panel C as a 3D volume projection, where the 532 nm channel is pseudo colored light blue for improved visualization. Signal localization, intensity, and distribution are consistent with those observed in widefield, widefield SIM, and confocal datasets, as well as with the unconjugated H2AX reference condition (Cat. ID# PA-MM-000002), satisfying CDx go/no-go criteria for analytical sensitivity and localization specificity. Collectively, these data support Airyscan as a transitional modality within Identifyn®'s step-down imaging methodology, enabling progression to higher-resolution approaches required for chromatin-level structural characterization.

EVIDENCE TIER	Airyscan
INSTRUMENT	ZEISS LSM 980
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 532; 647; 750
METADATA VALUES	59

STRUCTURED ACQUISITION METADATA / AIRYSCAN

The values below were deterministically extracted from the preserved Identifyn method PDF text. They remain source-documented metadata, not new BENNETT measurements.

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:
Objective	Plan-Apochromat 63X/1.40 oil DIC M27
Z-Stack	321 Slices (3.10 µm)
Channels	3
Scaling (Per Pixel)	0.03530 µm X 0.03530 µm X 0.01000 µm
Image size (Pixels)	698 X 1033 | 299 X 269
Image Size (Scaled)	24.64 µm X 36.46 µm | 10.55 µm X 9.49 µm
Bit Depth	16 Bit
Image Center Position	X: 58.51 µm, Y: 52.85 µm
Stage Position	X: 58.51 µm, Y: 52.85 µm
ROI Center Offset	X: 1.14 µm, Y: 0.00 µm
Reflector	None
Contrast Method	Fluorescence
Pinhole	5.00AU / 265µm | 5.00AU / 328µm | 5.00AU / 214µm
LMS MBS	MBS 488/639 & 405/730
LMS SBS	SBS LP 525 | SBS LP 640 | SBS SP 505
Laser Wavelength	488nm @1.50% | 639nm @0.1% | 405nm @0.2%
Laser Blanking	Enabled
Scan Mode	Frame
Scan Zoom	2.0
Rotation	0°
Sampling	2.00
Pixel Time	1.10µs
Frame Time	9.39s
LSM Scan Speed	6
Scan Direction	Bidirectional
Line Step	1
Averaging	4
Excitation Wavelength	528 | 653 | 353
Emission Wavelength	553 | 668 | 465
Effective NA	1.4
Detection Wavelength	530 - 582 | 643 - 735 | 422 - 477
Imaging Device	Airyscan

FIELD	SOURCE-DOCUMENTED VALUE
Detector Type	GaAsP-PMT | GaAsp-PMT
Detector Gain	800 V | 750 V
Detector Offset	0
Detector Digital Gain	1.0
Super Resolution	9.6 (3D, Auto) | 10.0 (3D, Auto) | 9.1 (3D, Auto)
3D Volume Projection	Precise
Appearance	Threshold 3%, Ramp 97%, Maximum 100%
Background	Black
Light	Brightness 100%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping
Projection	View Angle 35°, Scale Z 10%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

SOURCE METHOD

The text below is carried from the preserved Identifyn source record. Typography and pagination have been normalized for this preservation dossier; scientific wording has not been editorially rewritten.

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7)
Cat ID# - DC 000056

Rabbit anti-Human BRCA1 Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647, IgG - (BLR023N)
Cat ID# - DC 000046 - (Acquired but not Shown in Analysis)

H2AX, a variant of the histone protein H2A, and its primary function relates to the organization and structure of chromatin, as well as the cellular response to DNA damage. Here are the essential functions and roles of H2AX:

Nucleosome Formation: H2AX, like other histone proteins, contributes to the formation of nucleosomes. A nucleosome is the fundamental unit of chromatin, consisting of DNA wrapped around a core of histone proteins (including two molecules each of H2A, H2B, H3, and H4). This organization plays a crucial role in compacting DNA within the cell nucleus and regulating gene expression by controlling access to DNA.

DNA Damage Response (DDR): One of H2AX's key functions, distinct from those of other histone proteins, is its function in the DNA damage response. When DNA suffers double-strand breaks (DSBs), H2AX is phosphorylated at a specific serine residue (serine 139), leading to the formation of γ-H2AX (gamma H2AX). This phosphorylation serves as an early signal of DNA damage.

Recruitment of DNA Repair Proteins: Phosphorylation of H2AX to form γ-H2AX creates a binding site for various proteins involved in DNA repair. It helps recruit proteins involved in the recognition, signaling, and repair of DNA damage, such as MDC1 (Mediator of DNA Damage Checkpoint 1), 53BP1 (p53-binding protein 1), BRCA1 (Breast Cancer 1), and others. These proteins work together to repair the damage and maintain genomic stability.

Chromatin Remodeling: Through its role in signaling and repair, H2AX plays a role in chromatin remodeling during the DNA damage response. The formation of γ-H2AX marks regions where chromatin structure is altered to allow access for repair proteins and facilitate the repair process.

H2AX's functions are fascinating studies in molecular biology. They involve a complex interplay of structural and signaling roles. Its structural role in chromatin and its specialized role in signaling and facilitating the DNA damage response, particularly in double-strand breaks, are of immense interest. Through its phosphorylation to form γ-H2AX, it acts as a key marker for DNA damage and plays an essential role in the recruitment and organization of proteins implicated in the DNA repair process.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2μM Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.

The second primary antibody, Rabbit anti-Human BRCA1 Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647, IgG - (BLR023N) was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI (cat# P36962).

Control Data - Primary Unconjugated Antibody

Microscope

Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:

Z Stack - (3D) - Panel A, Z-plane 156 0f 321

Z-Stack = 321 Slices (3.10 µm)

Channels = 3
Scaling (Per Pixel) = 35.30 nm X 35.30 nm X 10.00 nm
Image size (Pixels) = 698 X 1033
Image Size (Scaled) = 24.64 µm X 36.46 µm
Bit Depth = 16 Bit
Image Center Position = X: 58.51 µm, Y: 52.85 µm
Stage Position = X: 58.51 µm, Y: 52.85 µm
ROI Center Offset = X: 1.14 µm, Y: 0.00 µm

Z Stack - (3D) - Panels B and C

Z-Stack = 321 Slices (3.10 µm)

Channels = 3
Scaling (Per Pixel) = 35.30 nm X 35.30 nm X 10.00 nm
Image size (Pixels) = 299 X 269
Image Size (Scaled) = 10.55 µm X 9.49 µm
Bit Depth = 16 Bit
Image Center Position = X: 58.51 µm, Y: 52.85 µm
Stage Position = X: 58.51 µm, Y: 52.85 µm
ROI Center Offset = X: 1.14 µm, Y: 0.00 µm

532 -

Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.00AU / 265µm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = SBS LP 525
Laser Wavelength = 488nm @1.50%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.0
Rotation = 0°
Sampling = 2.00
Pixel Time = 1.10µs
Frame Time = 9.39s
LSM Scan Speed = 6
Scan Direction = Bidirectional
Line Step = 1
Averaging = 4
Excitation Wavelength = 528
Emission Wavelength = 553
Effective NA = 1.4
Detection Wavelength = 530 - 582
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 800 V
Detector Offset = 0
Detector Digital Gain = 1.0
Super Resolution = 9.6 (3D, Auto)

647 - (Channel Acquired but not shown in Analysis)

Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.00AU / 328µm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = SBS LP 640
Laser Wavelength = 639nm @0.1%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.0
Rotation = 0°
Sampling = 2.00
Pixel Time = 1.10µs
Frame Time = 9.39s
LSM Scan Speed = 6
Scan Direction = Bidirectional
Line Step = 1
Averaging = 4
Excitation Wavelength = 653
Emission Wavelength = 668
Effective NA = 1.4
Detection Wavelength = 643 - 735
Imaging Device = Airyscan
Detector Type = GaAsP-PMT
Detector Gain = 800 V
Detector Offset = 0
Detector Digital Gain = 1.0
Super Resolution = 10.0 (3D, Auto)

DAPI - (Channel Acquired but not shown in Analysis)

Reflector = None
Contrast Method = Fluorescence
Pinhole = 5.00AU / 214µm
LMS MBS = MBS 488/639 & 405/730
LMS SBS = SBS SP 505
Laser Wavelength = 405nm @0.2%
Laser Blanking = Enabled
Scan Mode = Frame
Scan Zoom = 2.0
Rotation = 0°
Sampling = 2.00
Pixel Time = 1.10µs
Frame Time = 9.39s
LSM Scan Speed = 6
Scan Direction = Bidirectional
Line Step = 1
Averaging = 4
Excitation Wavelength = 353
Emission Wavelength = 465
Effective NA = 1.4
Detection Wavelength = 422 - 477
Imaging Device = Airyscan
Detector Type = GaAsp-PMT
Detector Gain = 750 V
Detector Offset = 0
Detector Digital Gain = 1.0
Super Resolution = 9.1 (3D, Auto)

2-Dimensional View - Panels A and B

Panel A - Shows Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7) at z-plane 156 of 321 in default yellow with a white box signifying the region of interest. Panel B - uses the same z-plane to show the region of interest and changes the default color, yellow to white to improve visualization.

3D Image Processing

3D Volume Projection = Precise
Appearance = Threshold 3%, Ramp 97%, Maximum 100%
Background = Black
Light = Brightness 100%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping
Projection = View Angle 35°, Scale Z 10%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Summary

Airyscan imaging of the H2AX Alexa Fluor™ 532 Direct Conjugate following etoposide treatment shows a modest enhancement of the chromatin-associated signal relative to confocal imaging. Still, it does not yield quantitative or definitive structural resolution at this modality. Panel A shows a 2D optical section of the whole nucleus at z-plane 156 of 321, with a white box denoting the region of interest (ROI). The ROI is presented in Panel B as a 2D view, where the 532 nm channel is pseudocolored white to enhance visual contrast, and further examined in Panel C as a 3D volume projection, where the 532 nm channel is pseudocolored light blue for improved visualization. Signal localization, intensity, and distribution are consistent with those observed in widefield, widefield SIM, and confocal datasets, as well as with the unconjugated H2AX reference condition (Cat. ID# PA-MM-000002), satisfying CDx go/no-go criteria for analytical sensitivity and localization specificity. Collectively, these data support Airyscan as a transitional modality within Identifyn®'s step-down imaging methodology, enabling progression to higher-resolution approaches required for chromatin-level structural characterization.

Image Corrections

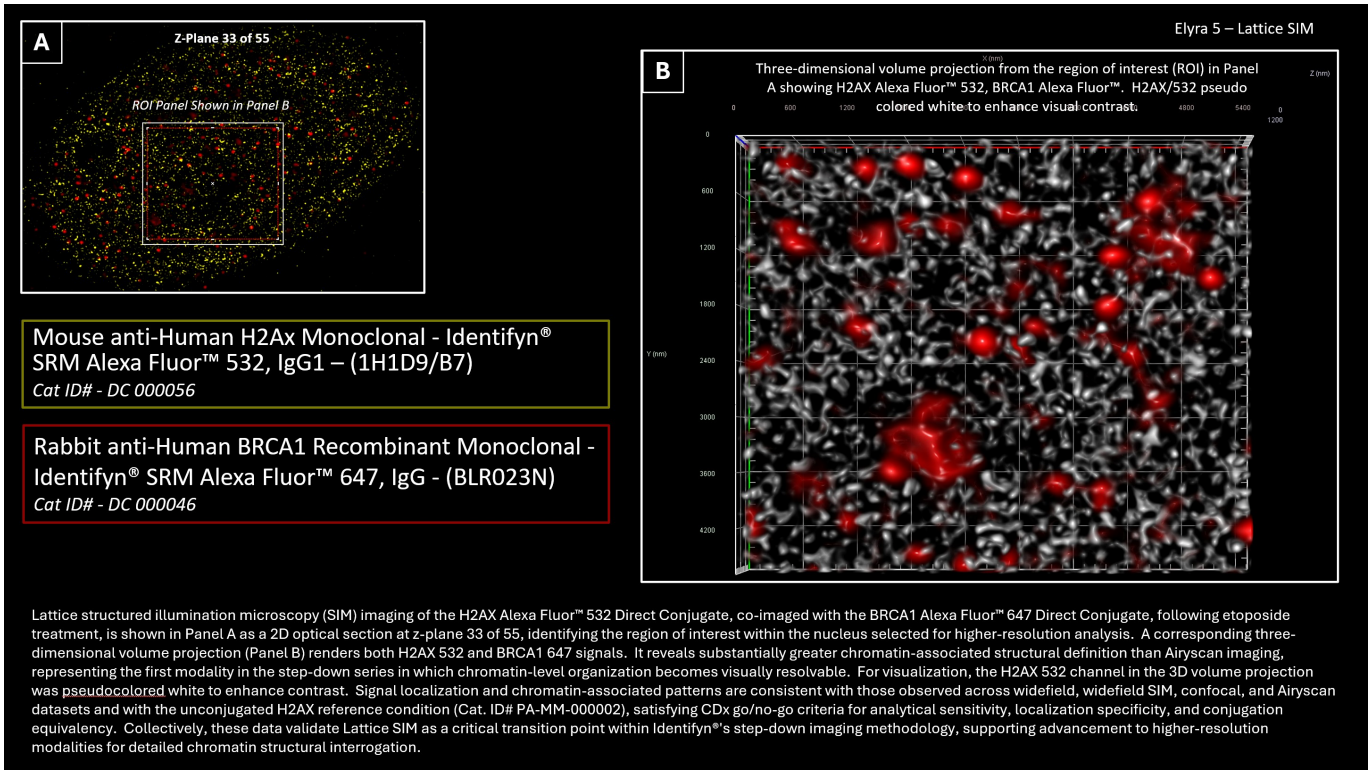
Identifyn® Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7) was pseudo-colored in Zen Software from yellow, the default color, to white, panel B, and light blue, panel C.

Image by J.K. Bennett
Image Analysis and Data Presentation by B.T. Bennett

SOURCE PROVENANCE

Catalog	DC-000056
Stage	Airyscan
Instrument	ZEISS LSM 980
Microscope configuration	Zeiss LSM 980 Confocal with NIR Detection, Axio Observer.Z1/7, using a Plan-Apochromat 63X/1.40 oil DIC M27, was used with the following parameters for each dye:
Structured source metadata values	59
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	0CA072ADA5D06C3EA2C9476C5DFB4F8776739B7467C4214E047E340C17510347
Method PDF SHA-256	1A1D7C03F1AA2E6486360DD0E962010938A1C1F0D27CAAB5C838933E34EEB506

ORIGINAL IMAGE EVIDENCE / SIM



Lattice structured illumination microscopy (SIM) imaging of the H2AX Alexa Fluor™ 532 Direct Conjugate, co-imaged with the BRCA1 Alexa Fluor™ 647 Direct Conjugate, following etoposide treatment, is shown in Panel A as a 2D optical section at z-plane 33 of 55, identifying the region of interest within the nucleus selected for higher-resolution analysis. A corresponding three-dimensional volume projection (Panel B) renders both H2AX 532 and BRCA1 647 signals. It reveals substantially greater chromatin-associated structural definition than Airyscan imaging, representing the first modality in the step-down series in which chromatin-level organization becomes visually resolvable. For visualization, the H2AX 532 channel in the 3D volume projection was pseudocolored white to enhance contrast. Signal localization and chromatin-associated patterns are consistent with those observed across widefield, widefield SIM, confocal, and Airyscan datasets and with the unconjugated H2AX reference condition (Cat. ID# PA-MM-000002), satisfying CDx go/no-go criteria for analytical sensitivity, localization specificity, and conjugation equivalency. Collectively, these data validate Lattice SIM as a critical transition point within Identifyn®'s step-down imaging methodology, supporting advancement to higher-resolution modalities for detailed chromatin structural interrogation.

EVIDENCE TIER	SIM
INSTRUMENT	ZEISS Elyra
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 532; 647
METADATA VALUES	54

STRUCTURED ACQUISITION METADATA / SIM

The values below were deterministically extracted from the preserved Identifyn method PDF text. They remain source-documented metadata, not new BENNETT measurements.

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Zeiss Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1.25X Tubelens, was used with the following parameters for each dye:
Objective	Plan-Apochromat 63X/1.40 oil DIC M27
Z-Stack	55 Slices (1.62 µm)
Channels	2
Scaling (Per Pixel)	0.02111 µm X 0.02111 µm X 0.03000 µm
Image size (Pixels)	1399 X 940 | 262 X 222
Image Size (Scaled)	29.54 µm X 19.85 µm | 5.53 µm X 4.69 µm
Bit Depth	16 Bit
Image Center Position	X: 60.62 µm, Y: 38.29 µm
Stage Position	X: 60.62 µm, Y: 38.29 µm
ROI Center Offset	X: 1.14 µm, Y: 0.00 µm
Reflectors	SR HE LBF 405/488/561/642
Beam Splitter	405, 488, 561, 642
Filter Excitation Wavelength	200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength	419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method	Fluorescence
Illumination Wavelength	488 | 640
Channel Name	T2-Axiocam 820 #2-SR | T1-Axiocam 820-SR
Excitation Wavelength	488 | 640
Emission Wavelength	525 | 729
Effective NA	1.4
Detection Wavelength	419 - 470, 503 - 546, 576 - 617, 657 - 800 | 503 - 546, 657 - 800
Imaging Device	Axiocam 820 #2 | Axiocam 820
Camera Adapter	1X
Exposure Time	150 ms | 120 ms
Depth of Focus	0.81 µm | 1.13 µm
Binning Mode	2,2
Laser Wavelength	488 nm @2.0% | 640 nm @2.0%
Frame Time	1.59 s
Scan Direction	Unidirectional
Grating	36.5 µm grating
Processing Dimension	3D
Result Sampling	4

FIELD	SOURCE-DOCUMENTED VALUE
Process Sampling	N/A
Algorithm	SIM
SIM Settings	Standard
Auto Sharpness	On
Sharpness	10.2282 (647), 11.2702 (532)
Fitting	Fast Fit
Normalization	Auto
3D Volume Projection	Precise
Appearance	Threshold 2% all channels, Ramp 98% all channels, Maximum 100% all channels
Background	Black
Light	Brightness 100%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping
Projection	View Angle 35°, Scale Z 50%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

SOURCE METHOD

The text below is carried from the preserved Identifyn source record. Typography and pagination have been normalized for this preservation dossier; scientific wording has not been editorially rewritten.

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7)
Cat ID# - DC 000056

Rabbit anti-Human BRCA1 Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647, IgG - (BLR023N)
Cat ID# - DC 000046

H2AX, a variant of the histone protein H2A, and its primary function relates to the organization and structure of chromatin, as well as the cellular response to DNA damage. Here are the essential functions and roles of H2AX:

Nucleosome Formation: H2AX, like other histone proteins, contributes to the formation of nucleosomes. A nucleosome is the fundamental unit of chromatin, consisting of DNA wrapped around a core of histone proteins (including two molecules each of H2A, H2B, H3, and H4). This organization plays a crucial role in compacting DNA within the cell nucleus and regulating gene expression by controlling access to DNA.

DNA Damage Response (DDR): One of H2AX's key functions, distinct from those of other histone proteins, is its function in the DNA damage response. When DNA suffers double-strand breaks (DSBs), H2AX is phosphorylated at a specific serine residue (serine 139), leading to the formation of γ-H2AX (gamma H2AX). This phosphorylation serves as an early signal of DNA damage.

Recruitment of DNA Repair Proteins: Phosphorylation of H2AX to form γ-H2AX creates a binding site for various proteins involved in DNA repair. It helps recruit proteins involved in the recognition, signaling, and repair of DNA damage, such as MDC1 (Mediator of DNA Damage Checkpoint 1), 53BP1 (p53-binding protein 1), BRCA1 (Breast Cancer 1), and others. These proteins work together to repair the damage and maintain genomic stability.

Chromatin Remodeling: Through its role in signaling and repair, H2AX plays a role in chromatin remodeling during the DNA damage response. The formation of γ-H2AX marks regions where chromatin structure is altered to allow access for repair proteins and facilitate the repair process.

H2AX's functions are fascinating studies in molecular biology. They involve a complex interplay of structural and signaling roles. Its structural role in chromatin and its specialized role in signaling and facilitating the DNA damage response, particularly in double-strand breaks, are of immense interest. Through its phosphorylation to form γ-H2AX, it acts as a key marker for DNA damage and plays an essential role in the recruitment and organization of proteins implicated in the DNA repair process.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2μM Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.

The second primary antibody, Rabbit anti-Human BRCA1 Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647, IgG - (BLR023N) was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI (cat# P36962).

Control Data - Primary Unconjugated Antibody

Microscope

Zeiss Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1.25X Tubelens, was used with the following parameters for each dye:

Z Stack - (3D) - Panel A, Z-plane 33 0f 55

Z-Stack = 55 Slices (1.62 µm)

Channels = 2
Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm
Image size (Pixels) = 1399 X 940
Image Size (Scaled) = 29.54 µm X 19.85 µm
Bit Depth = 16 Bit
Image Center Position = X: 60.62 µm, Y: 38.29 µm
Stage Position = X: 60.62 µm, Y: 38.29 µm
ROI Center Offset = X: 1.14 µm, Y: 0.00 µm

Z Stack - (3D) - Panel B

Z-Stack = 55 Slices (1.62 µm)

Channels = 2
Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm
Image size (Pixels) = 262 X 222
Image Size (Scaled) = 5.53 µm X 4.69 µm
Bit Depth = 16 Bit
Image Center Position = X: 60.62 µm, Y: 38.29 µm
Stage Position = X: 60.62 µm, Y: 38.29 µm
ROI Center Offset = X: 1.14 µm, Y: 0.00 µm

532 -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 488
Channel Name = T2-Axiocam 820 #2-SR
Excitation Wavelength = 488
Emission Wavelength = 525
Effective NA = 1.4
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820 #2
Camera Adapter = 1X
Exposure Time = 150 ms
Depth of Focus = 0.81 µm
Binning Mode = 2,2
Laser Wavelength = 488 nm @2.0%
Frame Time = 1.59 s
Scan Direction = Unidirectional
Grating = 36.5 µm grating

647 -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 640
Channel Name = T1-Axiocam 820-SR
Excitation Wavelength = 640
Emission Wavelength = 729
Effective NA = 1.4
Detection Wavelength = 503 - 546, 657 - 800
Imaging Device = Axiocam 820
Camera Adapter = 1X
Exposure Time = 120 ms
Depth of Focus = 1.13 µm
Binning Mode = 2,2
Laser Wavelength = 640 nm @2.0%
Frame Time = 1.59 s
Scan Direction = Unidirectional
Grating = 36.5 µm grating

Post Processing - SIM Processing

Processing Dimension = 3D
Result Sampling = 4
Process Sampling = N/A
Channels = 2
Algorithm = SIM
SIM Settings = Standard
Auto Sharpness = On
Sharpness = 10.2282 (647), 11.2702 (532)
Fitting = Fast Fit
Normalization = Auto

2-Dimensional View - Panels A and B

Panel A - Shows Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7) and Rabbit anti-Human BRCA1 Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647, IgG - (BLR023N) and at z-plane 33 of 55 in default yellow with a white box signifying the region of interest. Panel B - uses the same z-plane to show the region of interest and changes the default color, yellow to white, to improve visualization.

3D Image Processing

3D Volume Projection = Precise
Appearance = Threshold 2% all channels, Ramp 98% all channels, Maximum 100% all channels
Background = Black
Light = Brightness 100%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping
Projection = View Angle 35°, Scale Z 50%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Summary

Lattice structured illumination microscopy (SIM) imaging of the H2AX Alexa Fluor™ 532 Direct Conjugate, co-imaged with the BRCA1 Alexa Fluor™ 647 Direct Conjugate, following etoposide treatment, is shown in Panel A as a 2D optical section at z-plane 33 of 55, identifying the region of interest within the nucleus selected for higher-resolution analysis. A corresponding three-dimensional volume projection (Panel B) renders both H2AX 532 and BRCA1 647 signals. It reveals substantially greater chromatin-associated structural definition than Airyscan imaging, representing the first modality in the step-down series in which chromatin-level organization becomes visually resolvable. For visualization, the H2AX 532 channel in the 3D volume projection was pseudocolored white to enhance contrast. Signal localization and chromatin-associated patterns are consistent with those observed across widefield, widefield SIM, confocal, and Airyscan datasets and with the unconjugated H2AX reference condition (Cat. ID# PA-MM-000002), satisfying CDx go/no-go criteria for analytical sensitivity, localization specificity, and conjugation equivalency. Collectively, these data validate Lattice SIM as a critical transition point within Identifyn®'s step-down imaging methodology, supporting advancement to higher-resolution modalities for detailed chromatin structural interrogation.

Image Correction

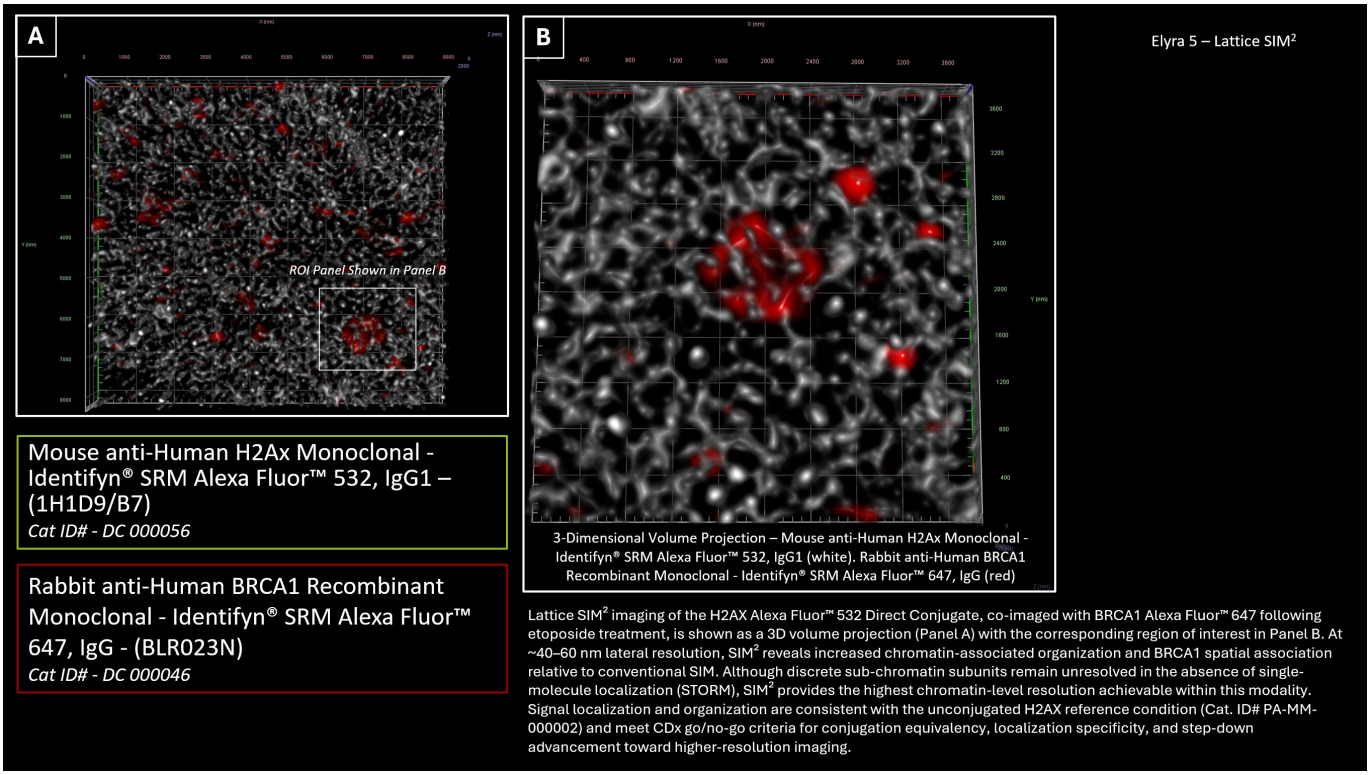
Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7) was pseudo colored white from its default yellow to white, enhancing visualization in panel B.

Image by P. Raut
Image Analysis and Data Presentation by B.T. Bennett

SOURCE PROVENANCE

Catalog	DC-000056
Stage	SIM
Instrument	ZEISS Elyra
Microscope configuration	Zeiss Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1.25X Tubelens, was used with the following parameters for each dye:
Structured source metadata values	54
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	BBE6D277B8A0B1FCFFCAF9D7FD173C0BDAF0F976A3F31B248264AA66AC1AFEA8
Method PDF SHA-256	59C70D1F633BD290CCD628C130BA1B13BC295A14B581DB46BE79ADAAEB4E30A6

ORIGINAL IMAGE EVIDENCE / SIM²



Lattice SIM² imaging of the H2AX Alexa Fluor™ 532 Direct Conjugate, co-imaged with BRCA1 Alexa Fluor™ 647 following etoposide treatment, is shown as a 3D volume projection (Panel A) with the corresponding region of interest in Panel B. At ~40–60 nm lateral resolution, SIM² reveals increased chromatin-associated organization and BRCA1 spatial association relative to conventional SIM. Although discrete sub-chromatin subunits remain unresolved in the absence of single-molecule localization (STORM), SIM² provides the highest chromatin-level resolution achievable within this modality. Signal localization and organization are consistent with the unconjugated H2AX reference condition (Cat. ID# PA-MM-000002) and meet CDx go/no-go criteria for conjugation equivalency, localization specificity, and step-down advancement toward higher-resolution imaging.

EVIDENCE TIER	SIM²
INSTRUMENT	ZEISS Elyra
CELL MODEL	HeLa
DETECTED DYES	405/DAPI; 488; 532; 647
METADATA VALUES	55

STRUCTURED ACQUISITION METADATA / SIM²

The values below were deterministically extracted from the preserved Identifyn method PDF text. They remain source-documented metadata, not new BENNETT measurements.

FIELD	SOURCE-DOCUMENTED VALUE
Platform / configuration	Zeiss Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1.25X Tubelens, was used with the following parameters for each dye:
Objective	Plan-Apochromat 63X/1.40 oil DIC M27
Z-Stack	107 Slices (3.18 µm)
Channels	2
Scaling (Per Pixel)	0.02111 µm X 0.02111 µm X 0.03000 µm
Image size (Pixels)	430 X 395 | 181 X 181
Image Size (Scaled)	9.08 µm X 8.34 µm | 3.82 µm X 3.82 µm
Bit Depth	16 Bit
Image Center Position	X: 60.62 µm, Y: 38.29 µm
Stage Position	X: 60.62 µm, Y: 38.29 µm
ROI Center Offset	X: 1.14 µm, Y: 0.00 µm
Reflectors	SR HE LBF 405/488/561/642
Beam Splitter	405, 488, 561, 642
Filter Excitation Wavelength	200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength	419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method	Fluorescence
Illumination Wavelength	488 | 640
Channel Name	T2-Axiocam 820 #2-SR | T1-Axiocam 820-SR
Excitation Wavelength	488 | 640
Emission Wavelength	525 | 729
Effective NA	1.4
Detection Wavelength	419 - 470, 503 - 546, 576 - 617, 657 - 800 | 503 - 546, 657 - 800
Imaging Device	Axiocam 820 #2 | Axiocam 820
Camera Adapter	1X
Exposure Time	150 ms | 120 ms
Depth of Focus	0.81 µm | 1.13 µm
Binning Mode	2,2
Laser Wavelength	488 nm @2.0% | 640 nm @2.0%
Frame Time	1.59 s
Scan Direction	Unidirectional
Grating	36.5 µm grating
Result Sampling	4
Process Sampling	4

FIELD	SOURCE-DOCUMENTED VALUE
Algorithm	SIM2
Input SNR	Low
Regularization Weight	0.0000
Iterations	3
Filter	No Filter
Fitting	Fast Fit
Normalization	Auto
3D Volume Projection	Precise
Appearance	Threshold 2% all channels, Ramp 98% all channels, Maximum 100% all channels
Background	Black
Light	Brightness 100%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping
Projection	View Angle 35°, Scale Z 50%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected) | View Angle 35°, Scale Z 10%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

SOURCE METHOD

The text below is carried from the preserved Identifyn source record. Typography and pagination have been normalized for this preservation dossier; scientific wording has not been editorially rewritten.

Identifyn Standards for Optical Microscopy Methods™

Identifyn® Primary Antibody

Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7)
Cat ID# - DC 000056

Rabbit anti-Human BRCA1 Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647, IgG - (BLR023N)
Cat ID# - DC 000046

H2AX, a variant of the histone protein H2A, and its primary function relates to the organization and structure of chromatin, as well as the cellular response to DNA damage. Here are the essential functions and roles of H2AX:

Nucleosome Formation: H2AX, like other histone proteins, contributes to the formation of nucleosomes. A nucleosome is the fundamental unit of chromatin, consisting of DNA wrapped around a core of histone proteins (including two molecules each of H2A, H2B, H3, and H4). This organization plays a crucial role in compacting DNA within the cell nucleus and regulating gene expression by controlling access to DNA.

DNA Damage Response (DDR): One of H2AX's key functions, distinct from those of other histone proteins, is its function in the DNA damage response. When DNA suffers double-strand breaks (DSBs), H2AX is phosphorylated at a specific serine residue (serine 139), leading to the formation of γ-H2AX (gamma H2AX). This phosphorylation serves as an early signal of DNA damage.

Recruitment of DNA Repair Proteins: Phosphorylation of H2AX to form γ-H2AX creates a binding site for various proteins involved in DNA repair. It helps recruit proteins involved in the recognition, signaling, and repair of DNA damage, such as MDC1 (Mediator of DNA Damage Checkpoint 1), 53BP1 (p53-binding protein 1), BRCA1 (Breast Cancer 1), and others. These proteins work together to repair the damage and maintain genomic stability.

Chromatin Remodeling: Through its role in signaling and repair, H2AX plays a role in chromatin remodeling during the DNA damage response. The formation of γ-H2AX marks regions where chromatin structure is altered to allow access for repair proteins and facilitate the repair process.

H2AX's functions are fascinating studies in molecular biology. They involve a complex interplay of structural and signaling roles. Its structural role in chromatin and its specialized role in signaling and facilitating the DNA damage response, particularly in double-strand breaks, are of immense interest. Through its phosphorylation to form γ-H2AX, it acts as a key marker for DNA damage and plays an essential role in the recruitment and organization of proteins implicated in the DNA repair process.

Method

HeLa cells were grown on acid-etched #1.5H cover glass to ~50% confluency. DNA damage was induced via treatment with 2μM Etoposide-spiked complete growth media for 24 hours, followed by -20°C methanol fixation and blocked with Thermo Fisher Fish Serum Blocker cat#37527X3. The primary antibody, Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7), was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 3X in PBS.

The second primary antibody, Rabbit anti-Human BRCA1 Recombinant Monoclonal - Identifyn® SRM Alexa Fluor™ 647, IgG - (BLR023N) was incubated for 1 hour at room temperature at a dilution of 1:250. Cells were washed 5X in PBS and mounted using ProLong™ Diamond Antifade Mountant with DAPI (cat# P36962).

Control Data - Primary Unconjugated Antibody

Microscope

Zeiss Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1.25X Tubelens, was used with the following parameters for each dye:

Z Stack - (3D) - Panel A

Z-Stack = 107 Slices (3.18 µm)

Channels = 2
Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm
Image size (Pixels) = 430 X 395
Image Size (Scaled) = 9.08 µm X 8.34 µm
Bit Depth = 16 Bit
Image Center Position = X: 60.62 µm, Y: 38.29 µm
Stage Position = X: 60.62 µm, Y: 38.29 µm
ROI Center Offset = X: 1.14 µm, Y: 0.00 µm

Z Stack - (3D) - Panel B

Z-Stack = 107 Slices (3.18 µm)

Channels = 2
Scaling (Per Pixel) = 21.11 nm X 21.11 nm X 30.00 nm
Image size (Pixels) = 181 X 181
Image Size (Scaled) = 3.82 µm X 3.82 µm
Bit Depth = 16 Bit
Image Center Position = X: 60.62 µm, Y: 38.29 µm
Stage Position = X: 60.62 µm, Y: 38.29 µm
ROI Center Offset = X: 1.14 µm, Y: 0.00 µm

532 -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 488
Channel Name = T2-Axiocam 820 #2-SR
Excitation Wavelength = 488
Emission Wavelength = 525
Effective NA = 1.4
Detection Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Imaging Device = Axiocam 820 #2
Camera Adapter = 1X
Exposure Time = 150 ms
Depth of Focus = 0.81 µm
Binning Mode = 2,2
Laser Wavelength = 488 nm @2.0%
Frame Time = 1.59 s
Scan Direction = Unidirectional
Grating = 36.5 µm grating

647 -

Reflectors = SR HE LBF 405/488/561/642
Beam Splitter = 405, 488, 561, 642
Filter Excitation Wavelength = 200 - 405, 200 - 488, 200 - 561, 200 - 642
Filter Emission Wavelength = 419 - 470, 503 - 546, 576 - 617, 657 - 800
Contrast Method = Fluorescence
Illumination Wavelength = 640
Channel Name = T1-Axiocam 820-SR
Excitation Wavelength = 640
Emission Wavelength = 729
Effective NA = 1.4
Detection Wavelength = 503 - 546, 657 - 800
Imaging Device = Axiocam 820
Camera Adapter = 1X
Exposure Time = 120 ms
Depth of Focus = 1.13 µm
Binning Mode = 2,2
Laser Wavelength = 640 nm @2.0%
Frame Time = 1.59 s
Scan Direction = Unidirectional
Grating = 36.5 µm grating

Post Processing - SIM2 Processing

Processing Dimension - 3D
Result Sampling = 4
Process Sampling = 4
Channels = 2
Algorithm = SIM2
Input SNR = Low
Regularization Weight = 0.0000
Iterations = 3
Filter = No Filter
Fitting = Fast Fit
Normalization = Auto

3D Image Processing - Panel A

3D Volume Projection = Precise
Appearance = Threshold 2% all channels, Ramp 98% all channels, Maximum 100% all channels
Background = Black
Light = Brightness 100%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping
Projection = View Angle 35°, Scale Z 50%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

3D Image Processing - Panel B

3D Volume Projection = Precise
Appearance = Threshold 2% all channels, Ramp 98% all channels, Maximum 100% all channels
Background = Black
Light = Brightness 100%, Azimuth 80°, Elongation -130°, Enabled Directional Lighting, Enabled Tone Mapping
Projection = View Angle 35°, Scale Z 10%, Reverse Z-Axis NOT Selected, Stereo Anaglyph NOT Selected, Camera Separation 5% (deselected), Parallax Shift 0% (deselected)

Summary

Lattice SIM² imaging of the H2AX Alexa Fluor™ 532 Direct Conjugate, co-imaged with BRCA1 Alexa Fluor™ 647, following etoposide treatment, is shown as a 3D volume projection (Panel A and the region of interest in Panel B). At ~40-60 nm lateral resolution, SIM² reveals increased chromatin-associated organization and BRCA1 spatial association relative to conventional SIM. Although discrete sub-chromatin subunits remain unresolved without single-molecule localization (STORM), SIM² provides the highest chromatin-level resolution achievable within this modality. Signal localization and organization are consistent with the unconjugated H2AX reference condition (Cat. ID# PA-MM-000002), supporting step-down progression to higher-resolution imaging.

Image Correction

Mouse anti-Human H2Ax Monoclonal - Identifyn® SRM Alexa Fluor™ 532, IgG1 - (1H1D9/B7) was pseudo colored white from its default yellow to white, enhancing visualization.

Image by P. Raut
Image Analysis and Data Presentation by B.T. Bennett

SOURCE PROVENANCE

Catalog	DC-000056
Stage	SIM²
Instrument	ZEISS Elyra
Microscope configuration	Zeiss Elyra 5 Lattice SIM, AxioObserver.Z1/ 7 using Plan-Apochromat 63X/1.40 oil DIC M27, 1.25X Tubelens, was used with the following parameters for each dye:
Structured source metadata values	55
Metadata source	PRESERVED_SOURCE_METHOD_PDF_TEXT
Cell model	HeLa
Image SHA-256	342A325F03D893287F2054386536785215146423440755 1AB03280051FE94952
Method PDF SHA-256	C4616B3EA1338CD396E93AB5B784552E91F3B70C59552FC8CC13B5E22CAB8FB7